

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C07D 311/00, 311/78, 493/00, C12N 1/21, 15/74, C12P 15/00, 17/06, 17/16</b>	<b>A1</b>	(11) International Publication Number: <b>WO 95/08548</b>
		(43) International Publication Date: <b>30 March 1995 (30.03.95)</b>

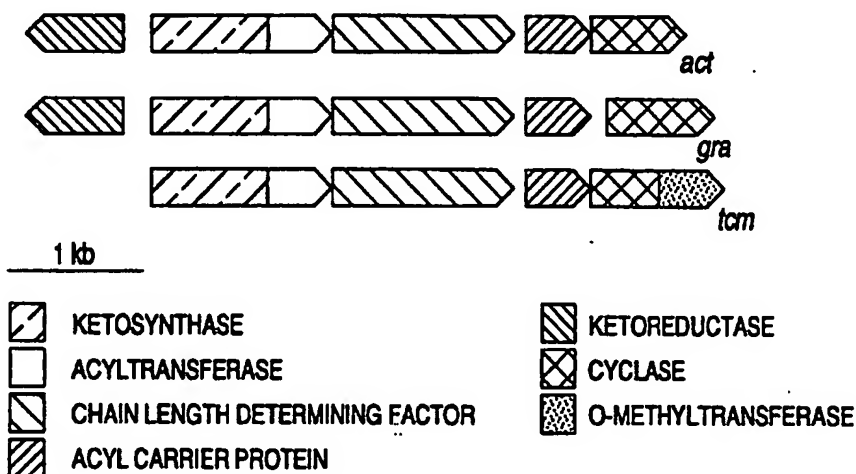
(21) International Application Number: **PCT/US94/10643**(22) International Filing Date: **20 September 1994 (20.09.94)**

## (30) Priority Data:

08/123,732	20 September 1993 (20.09.93)	US
08/164,301	8 December 1993 (08.12.93)	US
08/238,811	6 May 1994 (06.05.94)	US

(71) Applicants: **THE LELAND STANFORD JUNIOR UNIVERSITY [US/US];** Suite 350, 900 Welch Road, Palo Alto, CA 94304-1850 (US). **JOHN INNES CENTRE [GB/GB];** Colney Lane, Norwich NR4 7YH (GB).(72) Inventors: **KHOSLA, Chaitan;** 132 Peter Courtts Circle, Stanford, CA 94305 (US). **HOPWOOD, David, A.;** 244 Unthank Road, Norwich NR2 2AH (GB). **EBERT-KHOSLA, Suzanne;** 132 Peter Courtts Circle, Stanford, CA 94305 (US). **MCDANIEL, Robert;** 2891 Alma Street, No. D, Palo Alto, CA 94306 (US). **FU, Hong;** Chemical Engineering, Stauffer III, Stanford University, Stanford, CA 94305-5025 (US). **KAO, Camilla;** 19A Escondido Village, Stanford, CA 94305 (US).(74) Agent: **ROBINS, Roberta, L.;** Reed & Robins, 635 Bryant Street, Palo Alto, CA 94301 (US).(81) Designated States: **AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).**

## Published

*With international search report.  
With amended claims.*(54) Title: **RECOMBINANT PRODUCTION OF NOVEL POLYKETIDES**

## (57) Abstract

Novel polyketides and novel methods of efficiently producing both new and known polyketides, using recombinant technology, are disclosed. In particular, a novel host-vector system is described which is used to produce polyketide synthases which in turn catalyze the production of a variety of polyketides.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

5                    Recombinant Production of Novel PolyketidesDescriptionCross-Reference to Related Applications

10                    This application is a continuation-in-part of  
U.S. Patent Application Serial No. 08/164,301, filed  
December 8, 1993, which is a continuation-in-part of U.S.  
Application Serial No. 08/123,732, filed September 20,  
1993, from which priority is claimed pursuant to 35  
15 U.S.C. § 120, and which disclosures are hereby  
incorporated by reference in their entireties.

Technical Field

20                    The present invention relates generally to  
polyketides and polyketide synthases. In particular, the  
invention pertains to the recombinant production of  
polyketides using a novel host-vector system.

Background of the Invention

25                    Polyketides are a large, structurally diverse  
family of natural products. Polyketides possess a broad  
range of biological activities including antibiotic and  
pharmacological properties. For example, polyketides are  
represented by such antibiotics as tetracyclines and  
30 erythromycin, anticancer agents including daunomycin,  
immunosuppressants, for example FK506 and rapamycin, and  
veterinary products such as monensin and avermectin.  
Polyketides occur in most groups of organisms and are  
especially abundant in a class of mycelial bacteria, the  
35 actinomycetes, which produce various polyketides.

Polyketide synthases (PKSs) are multifunctional enzymes related to fatty acid synthases (FASs). PKSs catalyze the biosynthesis of polyketides through repeated (decarboxylative) Claisen condensations between  
5 acylthioesters, usually acetyl, propionyl, malonyl or methylmalonyl. Following each condensation, they introduce structural variability into the product by catalyzing all, part, or none of a reductive cycle comprising a ketoreduction, dehydration, and  
10 enoylreduction on the  $\beta$ -keto group of the growing polyketide chain. After the carbon chain has grown to a length characteristic of each specific product, it is released from the synthase by thiolysis or acyltransfer. Thus, PKSs consist of families of enzymes which work  
15 together to produce a given polyketide. It is the controlled variation in chain length, choice of chain-building units, and the reductive cycle, genetically programmed into each PKS, that contributes to the variation seen among naturally occurring polyketides.

20 Two general classes of PKSs exist. One class, known as Type I PKSs, is represented by the PKSs for macrolides such as erythromycin. These "complex" or "modular" PKSs include assemblies of several large multifunctional proteins carrying, between them, a set of  
25 separate active sites for each step of carbon chain assembly and modification (Cortes, J. et al. *Nature* (1990) 348:176; Donadio, S. et al. *Science* (1991) 252:675; MacNeil, D.J. et al. *Gene* (1992) 115:119). Structural diversity occurs in this class from variations  
30 in the number and type of active sites in the PKSs. This class of PKSs displays a one-to-one correlation between the number and clustering of active sites in the primary sequence of the PKS and the structure of the polyketide backbone.

35

The second class of PKSs, called Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs have a single set of iteratively used active sites (Bibb, M.J. et al. *EMBO J.* (1989) 8:2727; Sherman, D.H. et al. *EMBO J.* (1989) 8:2717; Fernandez-Moreno, M.A. et al. *J. Biol. Chem.* (1992) 267:19278).

*Streptomyces* is an actinomycete which is an abundant producer of aromatic polyketides. In each *Streptomyces* aromatic PKS so far studied, carbon chain assembly requires the products of three open reading frames (ORFs). ORF1 encodes a ketosynthase (KS) and an acyltransferase (AT) active site; ORF2 encodes a protein similar to the ORF1 product but lacking the KS and AT motifs; and ORF3 encodes a discrete acyl carrier protein (ACP).

*Streptomyces coelicolor* produces the blue-pigmented polyketide, actinorhodin. The actinorhodin gene cluster (*act*), has been cloned (Malpartida, F. and Hopwood, D.A. *Nature* (1984) 309:462; Malpartida, F. and Hopwood, D.A. *Mol. Gen. Genet.* (1986) 205:66) and completely sequenced (Fernandez-Moreno, M.A. et al. *J. Biol. Chem.* (1992) 267:19278; Hallam, S.E. et al. *Gene* (1988) 74:305; Fernandez-Moreno, M.A. et al. *Cell* (1991) 66:769; Caballero, J. et al. *Mol. Gen. Genet.* (1991) 230:401). The cluster encodes the PKS enzymes described above, a cyclase and a series of tailoring enzymes involved in subsequent modification reactions leading to actinorhodin, as well as proteins involved in export of the antibiotic and at least one protein that specifically activates transcription of the gene cluster. Other genes required for global regulation of antibiotic biosynthesis, as well as for the supply of starter (acetyl CoA) and extender (malonyl CoA) units for polyketide biosynthesis, are located elsewhere in the genome.

The act gene cluster from *S. coelicolor* has been used to produce actinorhodin in *S. parvulus*. Malpartida, F. and Hopwood, D.A. *Nature* (1984) 309:462. Bartel et al. *J. Bacteriol.* (1990) 172:4816-4826, recombina-  
5 tly produced aloesaponarin II using *S. galilaeus* transformed with an *S. coelicolor* act gene cluster consisting of four genetic loci, actI, actIII, actIV and actVII. Hybrid PKSs, including the basic act gene set but with ACP genes derived from granaticin, oxytetracycline, tetracenomycin and frenolicin PKSs, have  
10 also been designed which are able to express functional synthases. Khosla, C. et al. *J. Bacteriol.* (1993) 175:2197-2204. Hopwood, D.A. et al. *Nature* (1985) 314:642-644, describes the production of hybrid  
15 polyketides, using recombinant techniques. Sherman, D.H. et al. *J. Bacteriol.* (1992) 174:6184-6190, reports the transformation of various *S. coelicolor* mutants, lacking different components of the act PKS gene cluster, with the corresponding granaticin (gra) genes from *S.*  
20 *violaceoruber*, in trans.

However, no one to date has described the recombinant production of polyketides using genetically engineered host cells which substantially lack their entire native PKS gene clusters.

25

#### Summary of the Invention

The present invention provides for novel polyketides and novel methods of efficiently producing both new and known polyketides, using recombinant  
30 technology. In particular, a novel host-vector system is used to produce PKSs which in turn catalyze the production of a variety of polyketides. Such polyketides are useful as antibiotics, antitumor agents, immunosuppressants and for a wide variety of other  
35 pharmacological purposes.

Accordingly, in one embodiment, the invention is directed to a genetically engineered cell which expresses a polyketide synthase (PKS) gene cluster in its native, nontransformed state, the genetically engineered  
5 cell substantially lacking the entire native PKS gene cluster.

In another embodiment, the invention is directed to the genetically engineered cell as described above, wherein the cell comprises:

10 (a) a replacement PKS gene cluster which encodes a PKS capable of catalyzing the synthesis of a polyketide; and

(b) one or more control sequences operatively linked to the PKS gene cluster, whereby the genes in the  
15 gene cluster can be transcribed and translated in the genetically engineered cell,

with the proviso that when the replacement PKS gene cluster comprises an entire PKS gene set, at least one of the PKS genes or control elements is heterologous  
20 to the cell.

In particularly preferred embodiments, the genetically engineered cell is *Streptomyces coelicolor*, the cell substantially lacks the entire native actinorhodin PKS gene cluster and the replacement PKS  
25 gene cluster comprises a first gene encoding a PKS ketosynthase and a PKS acyltransferase active site (KS/AT), a second gene encoding a PKS chain length determining factor (CLF), and a third gene encoding a PKS acyl carrier protein (ACP).

30 In another embodiment, the invention is directed to a method for producing a recombinant polyketide comprising:

(a) providing a population of cells as described above; and  
35



(b) culturing the population of cells under conditions whereby the replacement PKS gene cluster present in the cells, is expressed.

In still another embodiment, the invention is directed to a method for producing a recombinant polyketide comprising:

a. inserting a first portion of a replacement PKS gene cluster into a donor plasmid and inserting a second portion of a replacement PKS gene cluster into a recipient plasmid, wherein the first and second portions collectively encode a complete replacement PKS gene cluster, and further wherein:

i. the donor plasmid expresses a gene which encodes a first selection marker and is capable of replication at a first, permissive temperature and incapable of replication at a second, non-permissive temperature;

ii. the recipient plasmid expresses a gene which encodes a second selection marker; and

iii. the donor plasmid comprises regions of DNA complementary to regions of DNA in the recipient plasmid, such that homologous recombination can occur between the first portion of the replacement PKS gene cluster and the second portion of the replacement gene cluster, whereby a complete replacement gene cluster can be generated;

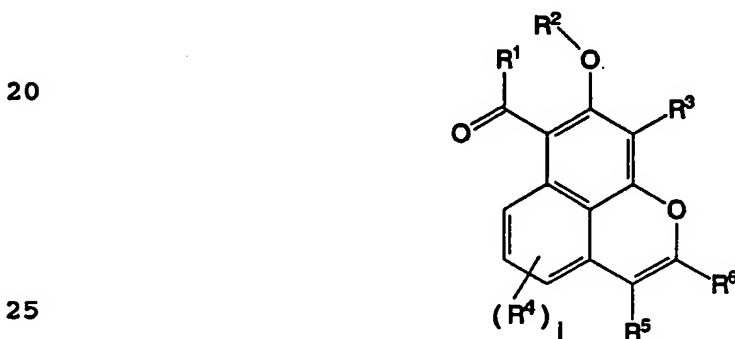
b. transforming the donor plasmid and the recipient plasmid into a host cell and culturing the transformed host cell at the first, permissive temperature and under conditions which allow the growth of host cells which express the first and/or the second selection markers, to generate a first population of cells;

c. culturing the first population of cells at the second, non-permissive temperature and under

conditions which allow the growth of cells which express the first and/or the second selection markers, to generate a second population of cells which includes host cells which contain a recombinant plasmid comprising a complete PKS replacement gene cluster;

- d. transferring the recombinant plasmid from the second population of cells into the genetically engineered cell of claim 1 to generate transformed genetically engineered cells; and
- e. culturing the transformed genetically engineered cells under conditions whereby the replacement PKS gene cluster present in the cells is expressed.

In yet another embodiment, the invention is directed to a polyketide compound having the structural formula (I)



wherein:

$R^1$  is selected from the group consisting of hydrogen and lower alkyl and  $R^2$  is selected from the group consisting of hydrogen, lower alkyl and lower alkyl ester, or wherein  $R^1$  and  $R^2$  together form a lower

alkylene bridge optionally substituted with one to four hydroxyl or lower alkyl groups;

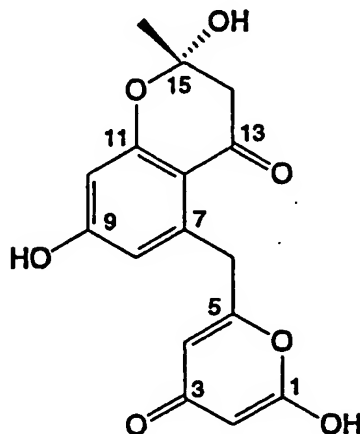
$R^3$  and  $R^5$  are independently selected from the group consisting of hydrogen, halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro;

$R^4$  is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro;

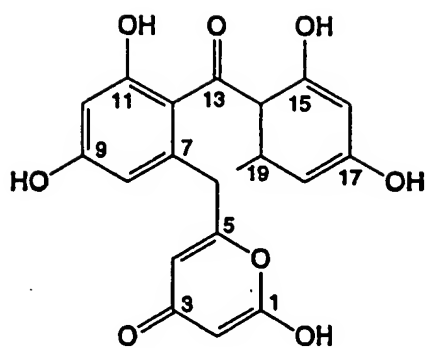
$R^6$  is selected from the group consisting of hydrogen, lower alkyl, and  $-\text{CHR}^7-(\text{CO})\text{R}^8$  where  $R^7$  and  $R^8$  are independently selected from the group consisting of hydrogen and lower alkyl; and

$i$  is 1, 2 or 3.

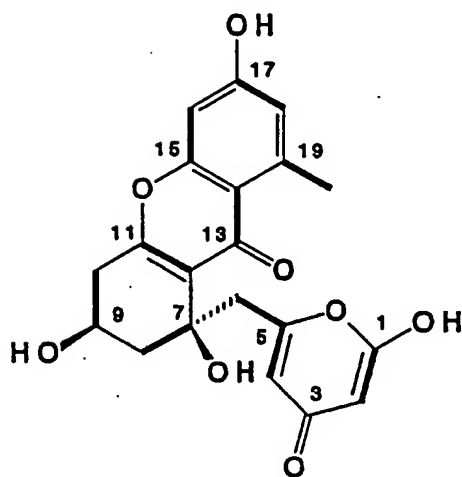
In another embodiment, the invention related to novel polyketides having the structures



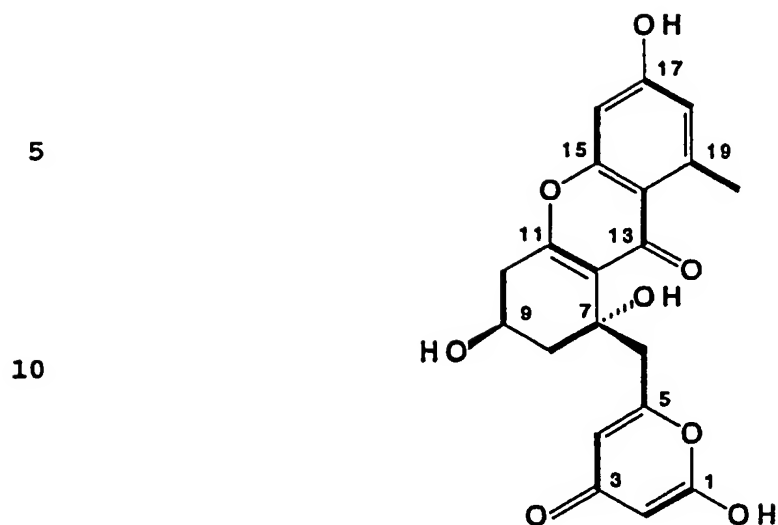
SEK4 (12)



SEK15 (13)

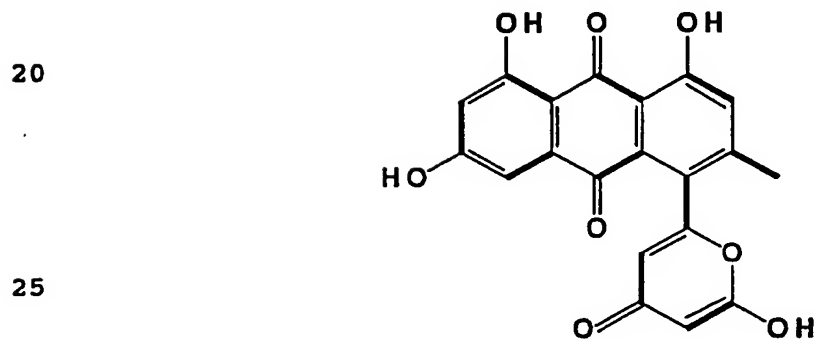


RM20b (14)



RM20c (15)

or

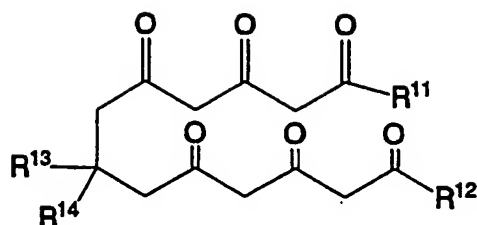


SEK15b (16)

30

In another embodiment, the invention is directed to a polyketide compound formed by catalytic cyclization of an enzyme-bound ketide having the structure (II)

35



wherein:

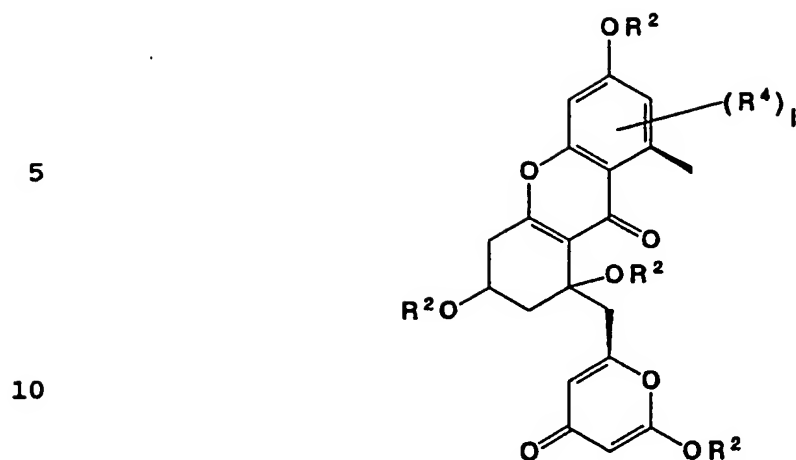
R<sup>11</sup> is selected from the group consisting of methyl, -CH<sub>2</sub>(CO)CH<sub>3</sub> and -CH<sub>2</sub>(CO)CH<sub>2</sub>(CO)CH<sub>3</sub>;

R<sup>12</sup> is selected from the group consisting of -S-E and -CH<sub>2</sub>(CO)-S-E, wherein E represents a polyketide synthase produced by the genetically engineered cells above; and

one of R<sup>13</sup> and R<sup>14</sup> is hydrogen and the other is hydroxyl, or R<sup>13</sup> and R<sup>14</sup> together represent carbonyl.

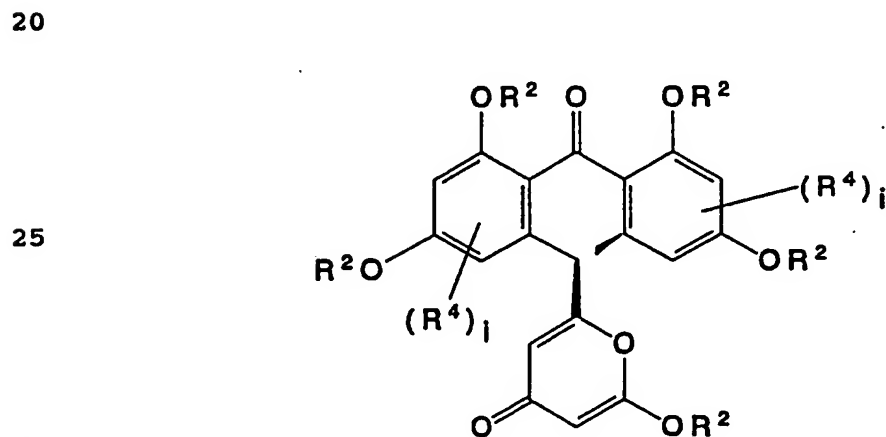
In still another embodiment, the invention is directed to a method for producing an aromatic polyketide, comprising effecting cyclization of an enzyme-bound ketide having the structure (II), wherein cyclization is induced by the polyketide synthase.

In a further embodiment, the invention is directed to a polyketide compound having the structural formula (III)



15 wherein  $R^2$  and  $R^4$  are as defined above and  $i$  is 0, 1 or 2.

In another embodiment, the invention is directed to a polyketide compound having the structural formula (IV)



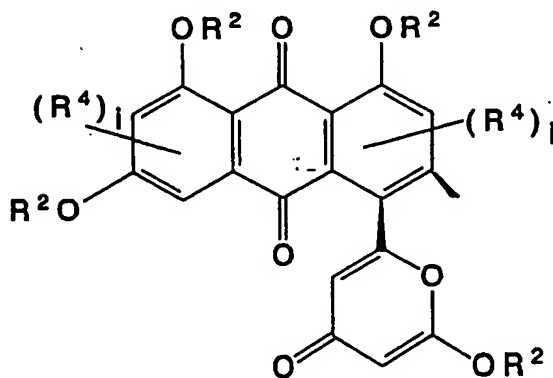
35 wherein  $R^2$ ,  $R^4$  and  $i$  are as defined above for structural formula (III).

In still another embodiment, the invention is directed to a polyketide compound having the structural formula (V)

5

10

15



wherein  $R^2$ ,  $R^4$  and  $i$  are as defined above for structural formula (III).

20

These and other embodiments of the subject invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

#### Brief Description of the Figures

25

Figure 1 shows the gene clusters for *act*, *gra*, and *tcm* PKSs and cyclases.

30

Figure 2 shows the strategy for making *S. coelicolor* CH999. Figure 2A depicts the structure of the *act* gene cluster present on the *S. coelicolor* CH1 chromosome. Figure 2B shows the structure of pLRemEts and Figure 2C shows the portion of the CH999 chromosome with the *act* gene cluster deleted.

Figure 3 is a diagram of plasmid pRM5.

35

Figure 4 schematically illustrates formation of aloesaponarin II (2) and its carboxylated analog, 3,8-



dihydroxy-1-methylanthraquinone-2-carboxylic acid (1) as described in Example 3.

Figure 5 provides the structures of actinorhodin (3), granaticin (4), tetracenomycin (5) and mutactin (6), referenced in Example 4.

Figure 6 schematically illustrates the preparation, via cyclization of the polyketide precursors, of aloesaponarin II (2), its carboxylated analog, 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (1), tetracenomycin (5) and new compound RM20 (9), as explained in Example 4, part (A).

Figure 7 schematically illustrates the preparation, via cyclization of the polyketide precursors, of frenolicin (7), nanomycin (8) and actinorhodin (3).

Figure 8 schematically illustrates the preparation, via cyclization of the polyketide precursors, of novel compounds RM20 (9), RM18 (10), RM18b (11), SEK4 (12), SEK15 (13), RM20b (14), RM20c (15) and SEK15b (16).

Figure 9 depicts the genetic model for the 6-deoxyerythronolide B synthase (DEBS).

Figure 10 shows the strategy for the construction of recombinant modular PKSs.

Figure 11 is a diagram of plasmid pCK7.

#### Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (Current Edition); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.);

*Oligonucleotide Synthesis* (N. Gait, ed., Current Edition); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., Current Edition); *Transcription and Translation* (B. Hames & S. Higgins, eds., Current Edition).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, reference to "a polyketide" includes mixtures of polyketides, reference to "a polyketide synthase" includes mixtures of polyketide synthases, and the like.

#### A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "replacement PKS gene cluster" is meant any set of PKS genes capable of producing a functional PKS when under the direction of one or more compatible control elements, as defined below, in a host cell transformed therewith. A functional PKS is one which catalyzes the synthesis of a polyketide. The term "replacement PKS gene cluster" encompasses one or more genes encoding for the various proteins necessary to catalyze the production of a polyketide. A "replacement PKS gene cluster" need not include all of the genes found in the corresponding cluster in nature. Rather, the gene cluster need only encode the necessary PKS components to catalyze the production of an active polyketide. Thus, as explained further below, if the gene cluster includes, for example, eight genes in its native state and only

three of these genes are necessary to provide an active polyketide, only these three genes need be present. Furthermore, the cluster can include PKS genes derived from a single species, or may be hybrid in nature with, e.g., a gene derived from a cluster for the synthesis of a particular polyketide replaced with a corresponding gene from a cluster for the synthesis of another polyketide. Hybrid clusters can include genes derived from both Type I and Type II PKSs. As explained above, Type I PKSs include several large multifunctional proteins carrying, between them, a set of separate active sites for each step of carbon chain assembly and modification. Type II PKSs, on the other hand, have a single set of iteratively used active sites. These classifications are well known. See, e.g., Hopwood, D.A. and Khosla, C. *Secondary metabolites: their function and evolution* (1992) Wiley Chichester (Ciba Foundation Symposium 171) p 88-112; Bibb, M.J. et al. *EMBO J.* (1989) 8:2727; Sherman, D.H. et al. *EMBO J.* (1989) 8:2717; Fernandez-Moreno, M.A. et al. *J. Biol. Chem.* (1992) 267:19278; Cortes, J. et al. *Nature* (1990) 348:176; Donadio, S. et al. *Science* (1991) 252:675; MacNeil, D.J. et al. *Gene* (1992) 115:119. Hybrid clusters are exemplified herein and are described further below. The genes included in the gene cluster need not be the native genes, but can be mutants or analogs thereof. Mutants or analogs may be prepared by the deletion, insertion or substitution of one or more nucleotides of the coding sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are described in, e.g., Sambrook et al., *supra*; *DNA Cloning*, Vols. I and II, *supra*; *Nucleic Acid Hybridization*, *supra*.

A "replacement PKS gene cluster" may also contain genes coding for modifications to the core polyketide catalyzed by the PKS, including, for example,

genes encoding hydroxylases, methylases or other alkylases, oxidases, reductases, glycotransferases, lyases, ester or amide synthases, and various hydrolases such as esterases and amidases.

5 As explained further below, the genes included in the replacement gene cluster need not be on the same plasmid or if present on the same plasmid, can be controlled by the same or different control sequences.

By "genetically engineered host cell" is meant  
10 a host cell where the native PKS gene cluster has been deleted using recombinant DNA techniques. Thus, the term would not encompass mutational events occurring in nature. A "host cell" is a cell derived from a procaryotic microorganism or a eucaryotic cell line  
15 cultured as a unicellular entity, which can be, or has been, used as a recipient for recombinant vectors bearing the PKS gene clusters of the invention. The term includes the progeny of the original cell which has been transfected. It is understood that the progeny of a  
20 single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be  
25 characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired PKS, are included in the definition, and are covered by the above terms.

The term "heterologous" as it relates to  
30 nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally associated with a region of a recombinant construct, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a  
35 nucleic acid construct is an identifiable segment of

nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by

5 sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene).

10 Similarly, a host cell transformed with a construct which is not normally present in the host cell would be considered heterologous for purposes of this invention. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used  
15 herein.

A "coding sequence" or a sequence which "encodes" a particular PKS, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in*  
20 *vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but  
25 is not limited to, cDNA from procaryotic or eucaryotic mRNA, genomic DNA sequences from procaryotic or eucaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

30 A "nucleic acid" sequence can include, but is not limited to, procaryotic sequences, eucaryotic mRNA, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. The term also captures sequences that include  
35 any of the known base analogs of DNA and RNA such as, but

not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-aminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. A transcription termination sequence will usually be located 3' to the coding sequence.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the desired gene is capable of being transcribed and translated.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding

sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

By "selection marker" is meant any genetic marker which can be used to select a population of cells which carry the marker in their genome. Examples of selection markers include: auxotrophic markers by which cells are selected by their ability to grow on minimal media with or without a nutrient or supplement, e.g., thymidine, diaminopimelic acid or biotin; metabolic markers by which cells are selected for their ability to grow on minimal media containing the appropriate sugar as the sole carbon source or the ability of cells to form colored colonies containing the appropriate dyes or chromogenic substrates; and drug resistance markers by which cells are selected by their ability to grow on media containing one or more of the appropriate drugs, e.g., tetracycline, ampicillin, kanamycin, streptomycin or nalidixic acid.

"Recombination" is a the reassortment of sections of DNA sequences between two DNA molecules. "Homologous recombination" occurs between two DNA molecules which hybridize by virtue of homologous or complementary nucleotide sequences present in each DNA molecule.

The term "alkyl" as used herein refers to a branched or unbranched saturated hydrocarbon group of 1 to 24 carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, t-butyl, octyl, decyl, tetradecyl, hexadecyl, eicosyl, tetracosyl and the like. Preferred alkyl groups herein contain 1 to 12 carbon

atoms. The term "lower alkyl" intends an alkyl group of one to six carbon atoms, preferably one to four carbon atoms.

5 The term "alkylene" as used herein refers to a difunctional saturated branched or unbranched hydrocarbon chain containing from 1 to 24 carbon atoms, and includes, for example, methylene ( $-\text{CH}_2-$ ), ethylene ( $-\text{CH}_2-\text{CH}_2-$ ), propylene ( $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ ), 2-methylpropylene [ $-\text{CH}_2-\text{CH}(\text{CH}_3)-\text{CH}_2-$ ], hexylene [ $-(\text{CH}_2)_6-$ ] and the like. "Lower  
10 alkylene" refers to an alkylene group of 1 to 6, more preferably 1 to 4, carbon atoms.

The term "alkoxy" as used herein intends an alkyl group bound through a single, terminal ether linkage; that is, an "alkoxy" group may be defined as -OR  
15 where R is alkyl as defined above. A "lower alkoxy" group intends an alkoxy group containing one to six, more preferably one to four, carbon atoms.

"Halo" or "halogen" refers to fluoro, chloro, bromo or iodo, and usually relates to halo substitution  
20 for a hydrogen atom in an organic compound. Of the halos, chloro and fluoro are generally preferred.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances  
25 where said event or circumstance occurs and instances where it does not. For example, the phrase "optionally substituted alkylene" means that an alkylene moiety may or may not be substituted and that the description includes both unsubstituted alkylene and alkylene where  
30 there is substitution.

#### B. General Methods

Central to the present invention is the discovery of a host-vector system for the efficient  
35 recombinant production of both novel and known



polyketides. In particular, the invention makes use of genetically engineered cells which have their naturally occurring PKS genes substantially deleted. These host cells can be transformed with recombinant vectors, encoding a variety of PKS gene clusters, for the production of active polyketides. The invention provides for the production of significant quantities of product at an appropriate stage of the growth cycle. The polyketides so produced can be used as therapeutic agents, to treat a number of disorders, depending on the type of polyketide in question. For example, several of the polyketides produced by the present method will find use as immunosuppressants, as anti-tumor agents, as well as for the treatment of viral, bacterial and parasitic infections. The ability to recombinantly produce polyketides also provides a powerful tool for characterizing PKSs and the mechanism of their actions.

More particularly, host cells for the recombinant production of the subject polyketides can be derived from any organism with the capability of harboring a recombinant PKS gene cluster. Thus, the host cells of the present invention can be derived from either procaryotic or eucaryotic organisms. However, preferred host cells are those constructed from the actinomycetes, a class of mycelial bacteria which are abundant producers of a number of polyketides. A particularly preferred genus for use with the present system is *Streptomyces*. Thus, for example, *S. ambofaciens*, *S. avermitilis*, *S. azureus*, *S. cinnamonensis*, *S. coelicolor*, *S. curacoi*, *S. erythraeus*, *S. fradiae*, *S. galilaeus*, *S. glaucescens*, *S. hygrosopicus*, *S. lividans*, *S. parvulus*, *S. peucetius*, *S. rimosus*, *S. roseofulvus*, *S. thermotolerans*, *S. violaceoruber*, among others, will provide convenient host cells for the subject invention, with *S. coelicolor* being preferred. (See, e.g., Hopwood, D.A. and Sherman, D.H.

Ann. Rev. Genet. (1990) 24:37-66; O'Hagan, D. *The Polyketide Metabolites* (Ellis Horwood Limited, 1991), for a description of various polyketide-producing organisms and their natural products.)

5           The above-described cells are genetically engineered by deleting the naturally occurring PKS genes therefrom, using standard techniques, such as by homologous recombination. (See, e.g., Khosla, C. et al. *Molec. Microbiol.* (1992) 6:3237). Exemplified herein is  
10 a genetically engineered *S. coelicolor* host cell. Native strains of *S. coelicolor* produce a PKS which catalyzes the biosynthesis of the aromatic polyketide actinorhodin (structure 3, Figure 5). The novel strain, *S. coelicolor* CH999 (Figure 2C and described in the examples), was  
15 constructed by deleting, via homologous recombination, the entire natural act cluster from the chromosome of *S. coelicolor* CH1 (Khosla, C. *Molec. Microbiol.* (1992) 6:3237), a strain lacking endogenous plasmids and carrying a stable mutation that blocks biosynthesis of  
20 another pigmented *S. coelicolor* antibiotic, undecylprodigiosin.

          The host cells described above can be transformed with one or more vectors, collectively encoding a functional PKS set. The vector(s) can include  
25 native or hybrid combinations of PKS subunits, or mutants thereof. As explained above, the replacement gene cluster need not correspond to the complete native gene cluster but need only encode the necessary PKS components to catalyze the production of a polyketide. For example,  
30 in each *Streptomyces* aromatic PKS so far studied, carbon chain assembly requires the products of three open reading frames (ORFs). ORF1 encodes a ketosynthase (KS) and an acyltransferase (AT) active site (KS/AT); as elucidated herein, ORF2 encodes a chain length  
35 determining factor (CLF), a protein similar to the ORF1

product but lacking the KS and AT motifs; and ORF3 encodes a discrete acyl carrier protein (ACP). Some gene clusters also code for a ketoreductase (KR) and a cyclase, involved in cyclization of the nascent polyketide backbone. (See Figure 1 for a schematic representation of three PKS gene clusters.) However, it has been found that only the KS/AT, CLF, and ACP, need be present in order to produce an identifiable polyketide. Thus, in the case of aromatic PKSs derived from *Streptomyces*, these three genes, without the other components of the native clusters, can be included in one or more recombinant vectors, to constitute a "minimal" replacement PKS gene cluster.

Furthermore, the recombinant vector(s) can include genes from a single PKS gene cluster, or may comprise hybrid replacement PKS gene clusters with, e.g., a gene for one cluster replaced by the corresponding gene from another gene cluster. For example, it has been found that ACPs are readily interchangeable among different synthases without an effect on product structure. Furthermore, a given KR can recognize and reduce polyketide chains of different chain lengths. Accordingly, these genes are freely interchangeable in the constructs described herein. Thus, the replacement clusters of the present invention can be derived from any combination of PKS gene sets which ultimately function to produce an identifiable polyketide.

Examples of hybrid replacement clusters include clusters with genes derived from two or more of the act gene cluster, frenolicin (*fren*), granaticin (*gra*), tetracenomycin (*tcm*), 6-methylsalicylic acid (*6-msas*), oxytetracycline (*otc*), tetracycline (*tet*), erythromycin (*ery*), griseusin, nanaomycin, medermycin, daunorubicin, tylosin, carbomycin, spiramycin, avermectin, monensin, nonactin, curamycin, rifamycin and candicidin synthase

gene clusters, among others. (For a discussion of various PKSs, see, e.g., Hopwood, D.A. and Sherman, D.H. *Ann. Rev. Genet.* (1990) 24:37-66; O'Hagan, D. *The Polyketide Metabolites* (Ellis Horwood Limited, 1991.)

5           More particularly, a number of hybrid gene clusters have been constructed herein, having components derived from the *act*, *fren*, *tcm* and *gra* gene clusters, as depicted in Tables 1 and 2. Several of the hybrid clusters were able to functionally express both novel and  
10 known polyketides in *S. coelicolor* CH999 (described above). However, other hybrid gene clusters, as described above, can easily be produced and screened using the disclosure herein, for the production of identifiable polyketides. For example, a library of  
15 randomly cloned ORF 1 and 2 homologs, from a collection of actinomycetes, could be constructed and screened for identifiable polyketides. Longer polyketides might also be cyclized by replacing, e.g., an *act*, *gra*, *fren* or *tcm* cyclase gene with a homolog from a PKS gene cluster which  
20 produces a chain of the correct length. Finally, a considerable degree of variability exists for non-acetate starter units among certain naturally occurring aromatic PKSs; thus, these units can also be used for obtaining novel polyketides via genetic engineering.

25           Additionally, the recombinant vectors can include genes from a modular PKS gene cluster. Such gene clusters are described in further detail below.

          The recombinant vectors, harboring the gene clusters described above, can be conveniently generated  
30 using techniques known in the art. For example, the PKS subunits of interest can be obtained from an organism that expresses the same, using recombinant methods, such as by screening cDNA or genomic libraries, derived from cells expressing the gene, or by deriving the gene from a  
35 vector known to include the same. The gene can then be

isolated and combined with other desired PKS subunits, using standard techniques. If the gene in question is already present in a suitable expression vector, it can be combined *in situ*, with, e.g., other PKS subunits, as  
5 desired. The gene of interest can also be produced synthetically, rather than cloned. The nucleotide sequence can be designed with the appropriate codons for the particular amino acid sequence desired. In general, one will select preferred codons for the intended host in  
10 which the sequence will be expressed. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; Jay et  
15 al. (1984) *J. Biol. Chem.* 259:6311.

Mutations can be made to the native PKS subunit sequences and such mutants used in place of the native sequence, so long as the mutants are able to function with other PKS subunits to collectively catalyze the  
20 synthesis of an identifiable polyketide. Such mutations can be made to the native sequences using conventional techniques such as by preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding a PKS subunit  
25 using restriction endonuclease digestion. (See, e.g., Kunkel, T.A. *Proc. Natl. Acad. Sci. USA* (1985) 82:448; Geisselsoder et al. *BioTechniques* (1987) 5:786.) Alternatively, the mutations can be effected using a mismatched primer (generally 10-20 nucleotides in length)  
30 which hybridizes to the native nucleotide sequence (generally cDNA corresponding to the RNA sequence), at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within  
35 relatively narrow limits and by keeping the mutant base

centrally located. Zoller and Smith, *Methods Enzymol.* (1983) 100:468. Primer extension is effected using DNA polymerase, the product cloned and clones containing the mutated DNA, derived by segregation of the primer  
5 extended strand, selected. Selection can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations. See, e.g., Dalbie-McFarland et al. *Proc. Natl. Acad. Sci USA* (1982) 79:6409. PCR  
10 mutagenesis will also find use for effecting the desired mutations.

The gene sequences which collectively encode a replacement PKS gene cluster, can be inserted into one or more expression vectors, using methods known to those of  
15 skill in the art. Expression vectors will include control sequences operably linked to the desired PKS coding sequence. Suitable expression systems for use with the present invention include systems which function in eucaryotic and procaryotic host cells. However, as  
20 explained above, procaryotic systems are preferred, and in particular, systems compatible with *Streptomyces spp.* are of particular interest. Control elements for use in such systems include promoters, optionally containing operator sequences, and ribosome binding sites.  
25 Particularly useful promoters include control sequences derived from PKS gene clusters, such as one or more act promoters. However, other bacterial promoters, such as those derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose, will also find use  
30 in the present constructs. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the  $\beta$ -lactamase (*bla*) promoter system, bacteriophage lambda PL, and T5. In addition, synthetic promoters, such as the *tac* promoter (U.S.

35

Patent No. 4,551,433), which do not occur in nature also function in bacterial host cells.

Other regulatory sequences may also be desirable which allow for regulation of expression of the  
5 PKS replacement sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in  
10 response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

Selectable markers can also be included in the recombinant expression vectors. A variety of markers are  
15 known which are useful in selecting for transformed cell lines and generally comprise a gene whose expression confers a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium. Such markers include, for example, genes which confer  
20 antibiotic resistance or sensitivity to the plasmid. Alternatively, several polyketides are naturally colored and this characteristic provides a built-in marker for selecting cells successfully transformed by the present constructs.

25 The various PKS subunits of interest can be cloned into one or more recombinant vectors as individual cassettes, with separate control elements, or under the control of, e.g., a single promoter. The PKS subunits can include flanking restriction sites to allow for the  
30 easy deletion and insertion of other PKS subunits so that hybrid PKSs can be generated. The design of such unique restriction sites is known to those of skill in the art and can be accomplished using the techniques described above, such as site-directed mutagenesis and PCR.

35

Using these techniques, a novel plasmid, pRM5, (Figure 3 and Example 2) was constructed as a shuttle vector for the production of the polyketides described herein. Plasmid pRM5 includes the *act* genes encoding the KS/AT (ORF1), CLF (ORF2) and ACP (ORF3) PKS subunits, flanked by *PacI*, *NsiI* and *XbaI* restriction sites. Thus, analogous PKS subunits, encoded by other PKS genes, can be easily substituted for the existing *act* genes. (See, e.g., Example 4, describing the construction of hybrid vectors using pRM5 as the parent plasmid). The shuttle plasmid also contains the *act* KR gene (*actIII*), the cyclase gene (*actVII*), and a putative dehydratase gene (*actIV*), as well as a *ColEI* replicon (to allow transformation of *E. coli*), an appropriately truncated *SCP2\** (low copy number) *Streptomyces* replicon, and the *actII*-ORF4 activator gene from the *act* cluster, which induces transcription from *act* promoters during the transition from growth phase to stationary phase in the vegetative mycelium. pRM5 carries the divergent *actI/actIII* promoter pair.

Methods for introducing the recombinant vectors of the present invention into suitable hosts are known to those of skill in the art and typically include the use of  $\text{CaCl}_2$  or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation. Once the PKSs are expressed, the polyketide producing colonies can be identified and isolated using known techniques. The produced polyketides can then be further characterized.

As explained above, the above-described recombinant methods also find utility in the catalytic biosynthesis of polyketides by large, modular PKSs. For example, 6-deoxyerythronolide B synthase (DEBS) catalyzes the biosynthesis of the erythromycin aglycone, 6-deoxyerythronolide B (17). Three open reading frames



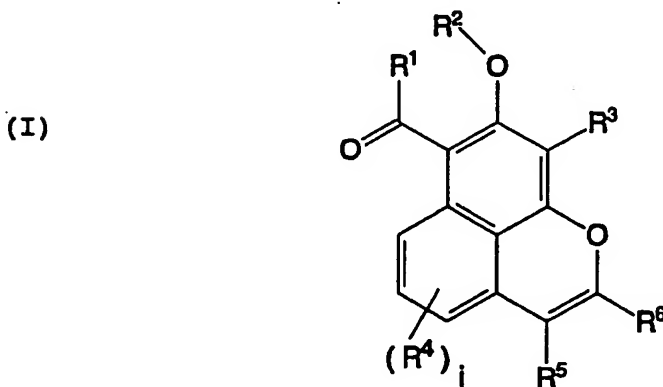
(*eryAI*, *eryAII*, and *eryAIII*) encode the DEBS polypeptides and span 32 kb in the *ery* gene cluster of the *Saccharopolyspora erythraea* genome. The genes are organized in six repeated units, each designated a "module." Each module encodes a set of active sites that, during polyketide biosynthesis, catalyzes the condensation of an additional monomer onto the growing chain. Each module includes an acyltransferase (AT),  $\beta$ -ketoacyl carrier protein synthase (KS), and acyl carrier protein (ACP) as well as a subset of reductive active sites ( $\beta$ -ketoreductase (KR), dehydratase (DH), enoyl reductase (ER)) (Figure 9). The number of reductive sites within a module corresponds to the extent of  $\beta$ -keto reduction in each condensation cycle. The thioesterase (TE) encoded at the end of module appears to catalyze lactone formation.

Due to the large sizes of *eryAI*, *eryAII*, and *eryAIII*, and the presence of multiple active sites, these genes can be conveniently cloned into a plasmid suitable for expression in a genetically engineered host cell, such as CH999, using an *in vivo* recombination technique. This technique, described in Example 5 and summarized in Figure 10, utilizes derivatives of the plasmid pMAK705 (Hamilton et al. (1989) *J. Bacteriol.* 171:4617) to permit *in vivo* recombination between a temperature-sensitive donor plasmid, which is capable of replication at a first, permissive temperature and incapable of replication at a second, non-permissive temperature, and recipient plasmid. The *eryA* genes thus cloned gave pCK7, a derivative of pRM5 (McDaniel et al. (1993) *Science* 262:1546). A control plasmid, pCK7f, was constructed to carry a frameshift mutation in *eryAI*. pCK7 and pCK7f possess a *ColEI* replicon for genetic manipulation in *E. coli* as well as a truncated SCP2\* (low copy number) *Streptomyces* replicon. These plasmids also contain the

divergent actI/actIII promoter pair and actII-ORF4, an activator gene, which is required for transcription from these promoters and activates expression during the transition from growth to stationary phase in the vegetative mycelium. High-level expression of PKS genes occurs at the onset of stationary phase of mycelial growth; the recombinant strains therefore produce "reporter" polyketides as secondary metabolites in a quasi-natural manner.

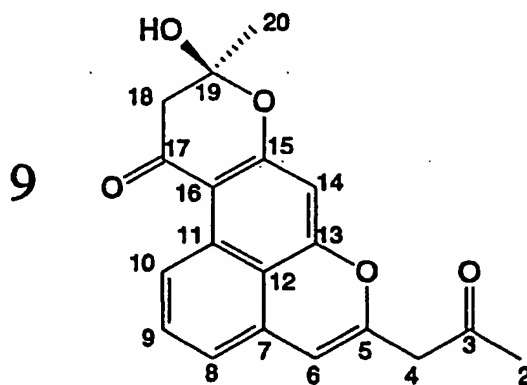
The method described above for producing polyketides synthesized by large, modular PKSs may be used to produce other polyketides as secondary metabolites such as sugars,  $\beta$ -lactams, fatty acids, aminoglycosides, terpenoids, non-ribosomal peptides, prostanoid hormones and the like.

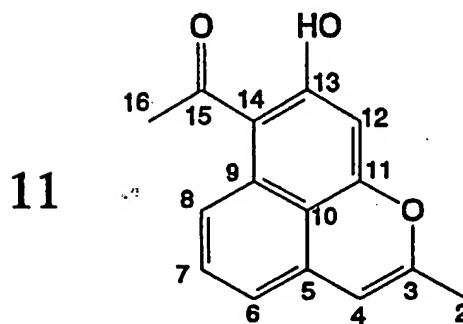
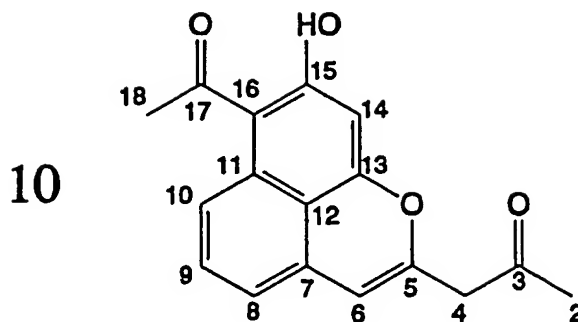
Using the above recombinant methods, a number of polyketides have been produced. These compounds have the general structure (I)



wherein  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^7$ ,  $R^8$  and  $i$  are as defined above. One group of such compounds are wherein:  $R^1$  is lower alkyl, preferably methyl;  $R^2$ ,  $R^3$  and  $R^6$  are hydrogen;  $R^6$  is  $-\text{CHR}^7-(\text{CO})-\text{R}^8$ ; and  $i$  is 0. A second

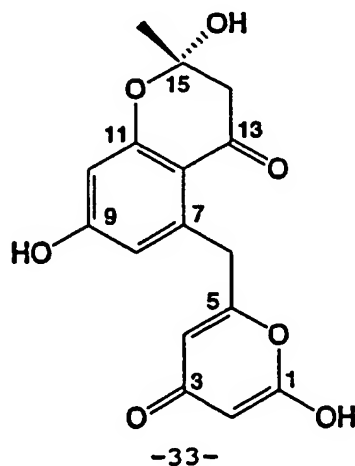
group of such compounds are wherein:  $R^1$  and  $R^6$  are lower alkyl, preferably methyl;  $R^2$ ,  $R^3$  and  $R^5$  are hydrogen; and  $i$  is 0. Still a third group of such compounds are wherein:  $R^1$  and  $R^2$  are linked together to form a lower alkylene bridge  $-\text{CHR}^9-\text{CHR}^{10}$  wherein  $R^9$  and  $R^{10}$  are independently selected from the group consisting of hydrogen, hydroxyl and lower alkyl, e.g.,  $-\text{CH}_2-\text{CHOH}-$ ;  $R^3$  and  $R^5$  are hydrogen;  $R^6$  is  $-\text{CHR}^7-(\text{CO})-\text{R}^8$  where  $R^8$  is hydrogen or lower alkyl, e.g.,  $-\text{CH}_2-(\text{CO})-\text{CH}_3$ ; and  $i$  is 0. Specific such compounds include the following compounds 9, 10 and 11 as follows:



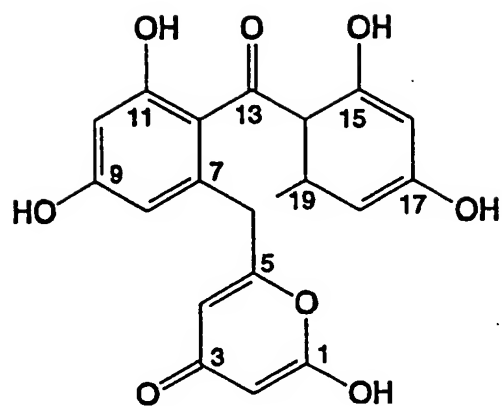


Other novel polyketides within the scope of the invention are those having the structure

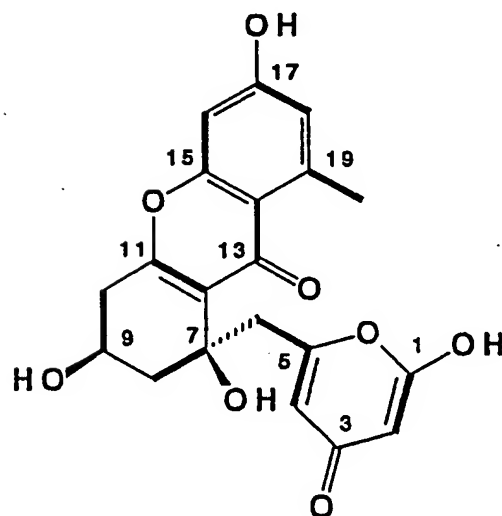
12



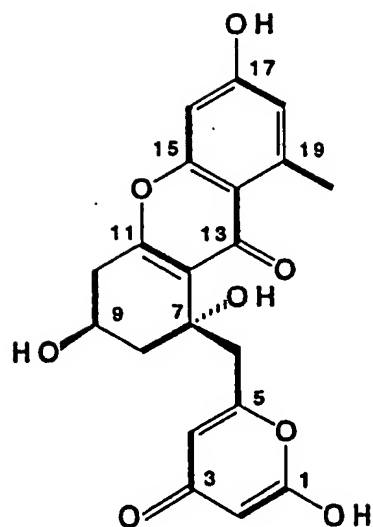
13



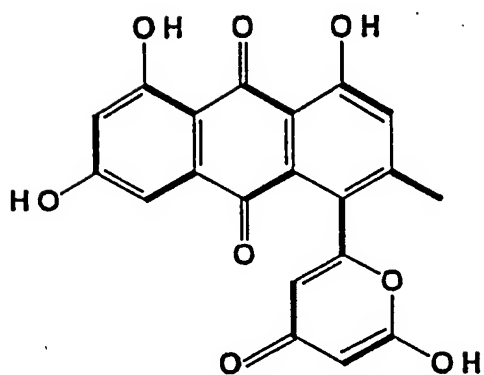
14



15



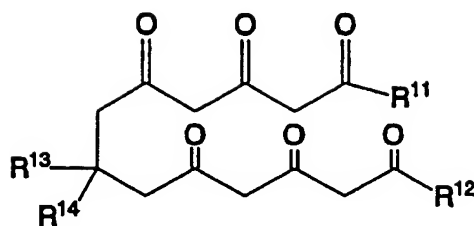
16



Preparation of compounds 9, 10, 11, 12, 13, 14, 15 and 16 is effected by cyclization of an enzyme-bound polyketide having the structure (II)

(II)

5

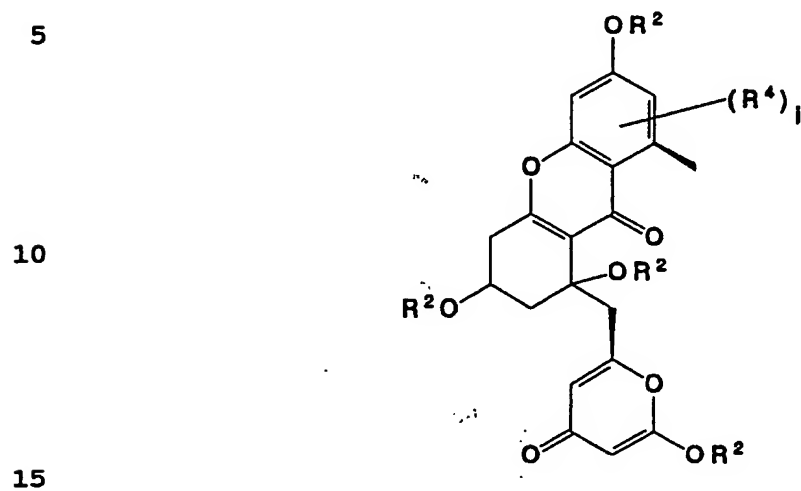


wherein  $R^{11}$ ,  $R^{12}$ ,  $R^{13}$  and  $R^{14}$  and E are as defined earlier  
 10 herein. Examples of such compounds include: a first  
 group wherein  $R^{11}$  is methyl and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ ; a  
 second group wherein  $R^{11}$  is  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{S}-\text{E}$ ; a  
 third group wherein  $R^{11}$  is  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ ; and a fourth group wherein  $R^{11}$  is  $-\text{CH}_2(\text{CO})\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$  (see Figure 8  
 15 for structural exemplification).

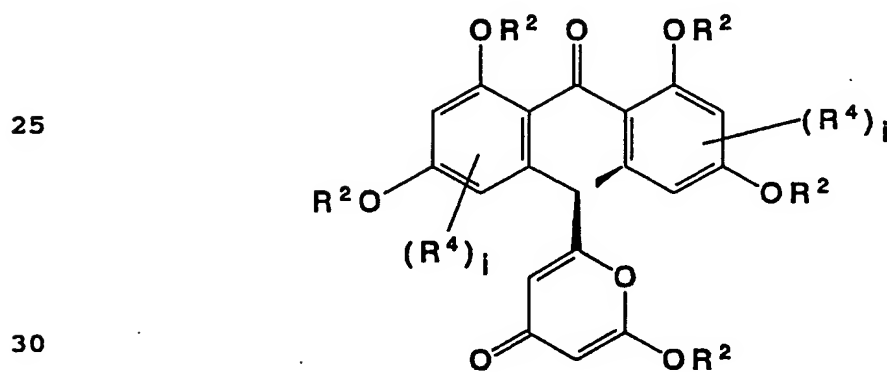
The remaining structures encompassed by generic  
 formula (I)--i.e., structures other than 9, 10 and 11--  
 may be prepared from structures 9, 10 or 11 using routine  
 20 synthetic organic methods well-known to those skilled in  
 the art of organic chemistry, e.g., as described by H.O.  
 House, Modern Synthetic Reactions, Second Edition (Menlo  
 Park, CA: The Benjamin/Cummings Publishing Company,  
 1972), or by J. March, Advanced Organic Chemistry:  
 25 Reactions, Mechanisms and Structure, 4th Ed. (New York:  
 Wiley-Interscience, 1992), the disclosures of which are  
 hereby incorporated by reference. Typically, as will be  
 appreciated by those skilled in the art, incorporation of  
 substituents on the aromatic rings will involve simple  
 30 electrophilic aromatic addition reactions. Structures 12  
 and 13 may be modified in a similar manner to produce  
 polyketides which are also intended to be within the  
 scope of the present invention.

35

In addition, the above recombinant methods have been used to produce polyketide compound having the general structure (III)

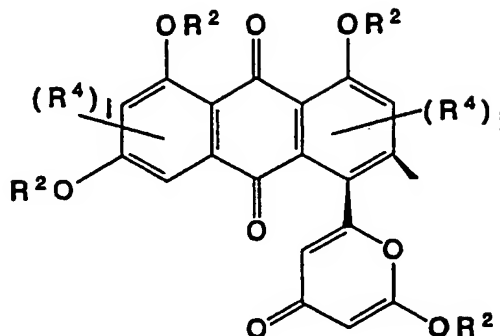


20 general structure (IV)





and general structure (V)



Particularly preferred compounds of structural formulas (III), (IV) and (V) are wherein:  $R^2$  is hydrogen and  $i$  is 0.

### C. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

### Materials and Methods

Bacterial strains, plasmids, and culture conditions. *S. coelicolor* CH999 was used as a host for transformation by all plasmids. The construction of this strain is described below. DNA manipulations were performed in *Escherichia coli* MC1061. Plasmids were

passed through *E. coli* ET12567 (*dam dcm hsdS*  $\text{Cm}^r$ ) (MacNeil, D.J. *J. Bacteriol.* (1988) 170:5607) to generate unmethylated DNA prior to transformation of *S. coelicolor*. *E. coli* strains were grown under standard conditions. *S. coelicolor* strains were grown on R2YE agar plates (Hopwood, D.A. et al. *Genetic manipulation of Streptomyces. A laboratory manual*. The John Innes Foundation: Norwich, 1985).

10                    Manipulation of DNA and organisms. Polymerase chain reaction (PCR) was performed using Taq polymerase (Perkin Elmer Cetus) under conditions recommended by the enzyme manufacturer. Standard *in vitro* techniques were used for DNA manipulations (Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (Current Edition)). *E. coli* was transformed with a Bio-Rad *E. Coli* Pulsing apparatus using protocols provided by Bio-Rad. *S. coelicolor* was transformed by standard procedures (Hopwood, D.A. et al. *Genetic manipulation of Streptomyces. A laboratory manual*. The John Innes Foundation: Norwich, 1985) and transformants were selected using 2 ml of a 500 mg/ml thiostrepton overlay.

25                    Construction of plasmids containing recombinant PKSs. All plasmids are derivatives of pRM5, described below. *fren* PKS genes were amplified via PCR with 5' and 3' restriction sites flanking the genes in accordance with the location of cloning sites on pRM5 (i.e. *PacI-NsiI* for ORF1, *NsiI-XbaI* for ORF2, and *XbaI-PstI* for ORF3). Following subcloning and sequencing, the amplified fragments were cloned in place of the corresponding fragments in pRM5 to generate the plasmids for transformation.

35

### Production and purification of polyketides.

For initial screening, all strains were grown at 30°C as confluent lawns on 10-30 plates each containing approximately 30 ml of agar medium for 6-8 days.

- 5 Additional plates were made as needed to obtain sufficient material for complete characterization. CH999 was a negative control when screening for potential polyketides. The agar was finely chopped and extracted with ethyl acetate/1% acetic acid or ethyl
- 10 acetate:methanol (4:1)/1% acetic acid. The concentrated extract was then flashed through a silica gel (Baker 40 mm) chromatography column in ethyl acetate/1% acetic acid. Alternatively, the extract was applied to a Florisil column (Fisher Scientific) and eluted with ethyl
- 15 acetate:ethanol:acetic acid (17:2:1). The primary yellow fraction was further purified via high-performance liquid chromatography (HPLC) using a 20-60% acetonitrile/water/1% acetic acid gradient on a preparative reverse phase (C-18) column (Beckman).
- 20 Absorbance was monitored at 280nm and 410nm. In general, the yield of purified product from these strains was approximately 10 mg/l for compounds 1 and 2 (Figure 4), and 5 mg/l for compounds 7 and 8 (Figure 7).

- SEK4, (12), was produced and purified as
- 25 follows. CH999/pSEK4 was grown on 90 agar plates (~ 34 ml/plate) at 30°C for 7 days. The agar was chopped and extracted with ethyl acetate/methanol (4/1) in the presence of 1% acetic acid (3 x 1000 ml). Following removal of the solvent under vacuum, 200 ml of ethyl
- 30 acetate containing 1% acetic acid were added. The precipitate was filtered and discarded, and the solvent was evaporated to dryness. The product mixture was applied to a Florisil column (Fisher Scientific), and eluted with ethyl acetate containing 3% acetic acid. The
- 35 first 100 ml fraction was collected, and concentrated

down to 5 ml. 1 ml methanol was added, and the mixture was kept at 4°C overnight. The precipitate was collected by filtration, and washed with ethyl acetate to give 850 mg of pure product.  $R_f = 0.48$  (ethyl acetate with 1% acetic acid). Results from NMR spectroscopy on SEK4 are reported in Table 4. FAB HRMS (NBA),  $M + H^+$ , calculated m/e 319.0818, observed m/e 319.0820.

To produce SEK15 (13) and SEK15b (16), CH999/pSEK15 was grown on 90 agar plates, and the product was extracted in the same manner as SEK4. The mixture was applied to a Florisil column (ethyl acetate with 5% acetic acid), and fractions containing the major products were combined and evaporated to dryness. The products were further purified using preparative C-18 reverse phase HPLC (Beckman) (mobile phase: acetonitrile/water = 1/10 to 3/5 gradient in the presence of 1% acetic acid). The yield of SEK15, (13), was 250 mg.  $R_f = 0.41$  (ethyl acetate with 1% acetic acid). Results from NMR spectroscopy on SEK4 are reported in Table 4. FAB HRMS (NBA),  $M + H^+$ , calculated m/e 385.0923, observed m/e 385.0920.

[1,2- $^{13}C_2$ ] acetate feeding experiments. Two 2 l flasks each containing 400ml of modified NMP medium (Strauch, E. et al. *Mol. Microbiol.* (1991) 5:289) were inoculated with spores of *S. coelicolor* CH999/pRM18, CH999/pSEK4 or CH999/pSEK15, and incubated in a shaker at 30 degrees C and 300 rpm. To each flask, 50mg of sodium [1,2- $^{13}C_2$ ] acetate (Aldrich) was added at 72 and 96 hrs. After 120 hrs, the cultures were pooled and extracted with two 500 ml volumes of ethyl acetate/1% acetic acid. The organic phase was kept and purification proceeded as described above.  $^{13}C$  NMR data indicate approximately a 2-3% enrichment for the CH999/pRM18 product; a 0.5-1% enrichment for SEK4 and a 1-2% enrichment for SEK15.

NMR Spectroscopy. All spectra were recorded on a Varian XL-400 except for HETCOR analysis of RM18 (10) (Figure 8), which was performed on a Nicolet NT-360. <sup>13</sup>C spectra were acquired with continuous broadband proton decoupling. For NOE studies of RM18 (10), the one-dimensional difference method was employed. All compounds were dissolved in DMSO-d<sub>6</sub> (Sigma, 99+ atom % D) and spectra were referenced internally to the solvent. Hydroxyl resonances were identified by adding D<sub>2</sub>O (Aldrich, 99 atom % D) and checking for disappearance of signal.

#### Example 1

##### Production of *S. coelicolor* CH999

An *S. coelicolor* host cell, genetically engineered to remove the native *act* gene cluster, and termed CH999, was constructed using *S. coelicolor* CH1 (Khosla, C. *Molec. Microbiol.* (1992) 6:3237), using the strategy depicted in Figure 2. (CH1 is derived from *S. coelicolor* B385 (Rudd, B.A.M. *Genetics of Pigmented Secondary Metabolites in Streptomyces coelicolor* (1978) Ph.D. Thesis, University of East Anglia, Norwich, England.) CH1 includes the *act* gene cluster which codes for enzymes involved in the biosynthesis and export of the polyketide antibiotic actinorhodin. The cluster is made up of the PKS genes, flanked by several post-PKS biosynthetic genes including those involved in cyclization, aromatization, and subsequent chemical tailoring (Figure 2A). Also present are the genes responsible for transcriptional activation of the *act* genes. The *act* gene cluster was deleted from CH1 using homologous recombination as described in Khosla, C. et al. *Molec. Microbiol.* (1992) 6:3237.

In particular, plasmid pLRermEts (Figure 2B) was constructed with the following features: a *ColEI*

replicon from pBR322, the temperature sensitive replicon from pSG5 (Muth, G. et al. *Mol. Gen. Genet.* (1989) 219:341), ampicillin and thiostrepton resistance markers, and a disruption cassette including a 2 kb *Bam*HI/*Xho*I fragment from the 5' end of the *act* cluster, a 1.5 kb *ermE* fragment (Khosla, C. et al. *Molec. Microbiol.* (1992) 6:3237), and a 1.9 kb *Sph*I/*Pst*I fragment from the 3' end of the *act* cluster. The 5' fragment extended from the *Bam*HI site 1 (Malpartida, F. and Hopwood, D.A. *Nature* (1984) 309:462; Malpartida, F. and Hopwood, D.A. *Mol. Gen. Genet.* (1986) 205:66) downstream to a *Xho*I site. The 3' fragment extended from *Pst*I site 20 upstream to *Sph*I site 19.2 (Fernandez-Moreno, M.A. et al. *J. Biol. Chem.* (1992) 267:19278). The 5' and 3' fragments (shown as hatched DNA in Figure 2) were cloned in the same relative orientation as in the *act* cluster. CH1 was transformed with pLRermEts. The plasmid was subsequently cured from candidate transformants by streaking non-selectively at 39°C. Several colonies that were lincomycin resistant, thiostrepton sensitive, and unable to produce actinorhodin, were isolated and checked via Southern blotting. One of them was designated CH999.

#### Example 2

##### Production of the Recombinant Vector pRM5

pRM5 (Figure 3) was the shuttle plasmid used for expressing PKSs in CH999. It includes a *ColE1* replicon to allow genetic engineering in *E. coli*, an appropriately truncated SCP2\* (low copy number) *Streptomyces* replicon, and the *actII*-ORF4 activator gene from the *act* cluster, which induces transcription from *act* promoters during the transition from growth phase to stationary phase in the vegetative mycelium. As shown in Figure 3, pRM5 carries the divergent *actI*/*actIII* promoter pair, together with convenient cloning sites to

facilitate the insertion of a variety of engineered PKS genes downstream of both promoters. pRM5 lacks the par locus of SCP2\*; as a result the plasmid is slightly unstable (approx. 2% loss in the absence of  
5 thiostrepton). This feature was deliberately introduced in order to allow for rapid confirmation that a phenotype of interest could be unambiguously assigned to the plasmid-borne mutant PKS. The recombinant PKSs from pRM5 are expressed approximately at the transition from  
10 exponential to stationary phase of growth, in good yields.

pRM5 was constructed as follows. A 10.5 kb *SphI/HindIII* fragment from pIJ903 (containing a portion  
15 of the fertility locus and the origin of replication of SCP2\* as well as the *colEI* origin of replication and the  $\beta$ -lactamase gene from pBR327) (Lydiate, D.J. Gene (1985) 35:223) was ligated with a 1.5 kb *HindIII/SphI* *tsr* gene cassette to yield pRM1. pRM5 was constructed by inserting the following two fragments between the unique  
20 *HindIII* and *EcoRI* sites of pRM1: a 0.3 kb *HindIII/HpaI*(blunt) fragment carrying a transcription terminator from phage fd (Khosla, C. et al. Molec. Microbiol. (1992) 6:3237), and a 10 kb fragment from the act cluster extending from the *NcoI* site (1 kb upstream  
25 of the actII-ORF4 activator gene) (Hallam, S.E. et al. Gene (1988) 74:305; Fernandez-Moreno, M.A. et al. Cell (1991) 66:769; Caballero, J.L. Mol. Gen. Genet. (1991) 230:401) to the *PstI* site downstream of the actI-VII-IV genes (Fernandez-Moreno, M.A. et al. J. Biol. Chem.  
30 (1992) 267:19278).

To facilitate the expression of any desired recombinant PKS under the control of the actI promoter (which is activated by the actII-ORF4 gene product), restriction sites for *PacI*, *NsiI*, *XbaI*, and *PstI* were  
35 engineered into the act DNA in intercistronic positions.

In pRM5, as well as in all other PKS expression plasmids described here, ORF1, 2, and 3 alleles were cloned between these sites as cassettes engineered with their own RBSs.

5                   In particular, in most naturally occurring aromatic polyketide synthase gene clusters in actinomycetes, ORF1 and ORF2 are translationally coupled. In order to facilitate construction of recombinant PKSs, the ORF1 and ORF2 alleles used here were cloned as  
10 independent (uncoupled) cassettes. For act ORF1, the following sequence was engineered into pRM5:

CCACCGGACGAACGCATCGATTAATTAAGGAGGACCATCATG, where the boldfaced sequence corresponds to upstream DNA from the actI region, TTAATTAA is the P<sub>ac</sub>I recognition site, and  
15 ATG is the start codon of act ORF1. The following sequence was engineered between act ORF1 and ORF2:  
NTGAATGCATGGAGGAGCCATCATG, where TGA and ATG are the stop and start codons of ORF1 and ORF2, respectively, ATGCAT is the NsiI recognition site, and the replacement of N (A  
20 in act DNA, A or G in alleles from other PKSs) with a C results in translational decoupling. The following sequence was engineered downstream of act ORF2:  
TAATCTAGA, where TAA is the stop codon, and TCTAGA is the XbaI recognition site. This allowed fusion of act ORF1  
25 and ORF2 (engineered as above) to an XbaI site that had been engineered upstream of act ORF3 (Khosla, C. et al. *Molec. Microbiol.* (1992) 6:3237). As a control, pRM2 was constructed, identical to pRM5, but lacking any of the engineered sequences. ORF1 and ORF2 in pRM2 are  
30 translationally coupled. Comparison of the product profiles of CH999/pRM2 and CH999/pRM5 revealed that the decoupling strategy described here had no detectable influence on product distribution or product levels.

35



Example 3Polyketides Produced using CH999 Transformed with pRM5

Plasmid pRM5 was introduced into *S. coelicolor* CH999 using standard techniques. (See, e.g., Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (Current Edition.) CH999 transformed with pRM5 produced a large amount of yellowish-brown material. The two most abundant products were characterized by NMR and mass spectroscopy as aloesaponarin II (2) (Bartel, P.L. et al. *J. Bacteriol.* (1990) 172:4816) and its carboxylated analog, 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (1) (Cameron, D.W. et al. *Liebigs Ann. Chem.* (1989) 7:699) (Figure 4). It is presumed that 2 is derived from 1 by non-enzymatic decarboxylation (Bartel, P.L. et al. *J. Bacteriol.* (1990) 172:4816). Compounds 1 and 2 were present in approximately a 1:5 molar ratio. Approximately 100 mg of the mixture could be easily purified from 1 l of culture. The CH999/pRM5 host-vector system was therefore functioning as expected to produce significant amounts of a stable, only minimally modified polyketide metabolite. The production of 1 and 2 is consistent with the proposed pathway of actinorhodin biosynthesis (Bartel, P.L. et al. *J. Bacteriol.* (1990) 172:4816). Both metabolites, like the actinorhodin backbone, are derived from a 16-carbon polyketide with a single ketoreduction at C-9.

When CH999 was transformed with pSEK4, identical to pRM5 except for replacement of a 140 bp *SphI/SalI* fragment within the *act KR* gene by the *SphI/SalI* fragment from pUC19, the resulting strain produced abundant quantities of the aromatic polyketide SEK4 (12). The exact structure of this product is slightly different from desoxyerythrolaccin (Bartel, P.L. et al. *J. Bacteriol.* (1990) 172:4816). However, in vivo isotopic labeling studies using 1,2-<sup>13</sup>C<sub>2</sub>- labeled acetate

confirmed that the polyketide backbone is derived from 8 acetates. Moreover, the aromatic region of the  $^1\text{H}$  spectrum, as well as the  $^{13}\text{C}$  NMR spectrum of this product, are consistent with a tricyclic structure similar to 1, but lacking any ketoreduction (see Table 4).

#### Example 4

#### Construction and Analysis of Hybrid Polyketide Synthases

10

#### A. Construction of hybrid PKSs including components from act, gra and tcm PKSs

Figure 1 shows the PKSs responsible for synthesizing the carbon chain backbones of actinorhodin (3), granaticin (4), and tetracenomycin (5) (structures shown in Figure 5) which contain homologous putative KS/AT and ACP subunits, as well as the ORF2 product. The act and gra PKSs also have KRs, lacking in the tcm PKS. Corresponding proteins from each cluster show a high degree of sequence identity. The percentage identities between corresponding PKS proteins in the three clusters are as follows: KS/AT: act/gr 76, act/tcm 64, gra/tcm 70; CLF: act/gr 60, act/tcm 58, gra/tcm 54; ACP: act/gr 60, act/tcm 43, gra/tcm 44. The act and gra PKSs synthesize identical 16-carbon backbones derived from 8 acetate residues with a ketoreduction at C-9 (Figure 6). In contrast, also as shown in Figure 6, the tcm polyketide backbone differs in overall carbon chain length (20 instead of 16 carbons), lack of any ketoreduction, and regiospecificity of the first cyclization, which occurs between carbons 9 and 14, instead of carbons 7 and 12 for act and gra.

In an attempt to generate novel polyketides, differing in a range of properties, as well as to elucidate aspects of the programming of aromatic PKSs, a

systematic series of minimal PKS gene clusters, using various permutations of the ORF1 (encoding the KS/AT subunit), ORF2 (encoding the CLF subunit) and ORF3 (encoding the ACP subunit) gene products from the *act*,  
5 *gra* and *tcm* gene clusters were cloned into pRM5 in place of the existing *act* genes, as shown in Table 1. The resulting plasmids were used to transform CH999 as above.

Analysis of the products of the recombinant PKSs containing various permutations among the KS/AT,  
10 ORF2 product, and ACP subunits of the PKSs (all constructs also containing the *act* KR, cyclase, and dehydratase genes) indicated that the synthases could be grouped into three categories (Table 1): those that did not produce any polyketide; those that produced compound  
15 1 (in addition to a small amount of 2); and those that produced a novel polyketide 9 (designated RM20) (Figure 6). The structure of 9 suggests that the polyketide backbone precursor of this molecule is derived from 10 acetate residues with a single ketoreduction at the C-9  
20 position.

In order to investigate the influence of the *act* KR on the reduction and cyclization patterns of a heterologous polyketide chain, pSEK15 was also constructed, which included *tcm* ORFs 1-3, but lacked the  
25 *act* KR. (The deletion in the *act* KR gene in this construct was identical to that in pSEK4.) Analysis of CH999/pSEK15 showed the 20 carbon chain product, SEK15 (13) which resembled, but was not identical to, tetracenomycin C or its shunt products. NMR spectroscopy  
30 was also consistent with a completely unreduced decaketide backbone (see Table 4).

All *act/gra* hybrids produced compound 1, consistent with the identical structures of the presumed actinorhodin and granaticin polyketides. In each case  
35 where a product could be isolated from a *tcm/act* hybrid,

the chain length of the polyketide was identical to that of the natural product corresponding to the source of ORF2. This implies that the ORF2 product, and not the ACP or KS/AT, controls carbon chain length. Furthermore, since all polyketides produced by the hybrids described here, except the ones lacking the KR (CH999/pSEK4 and CH999/pSEK15), underwent a single ketoreduction, it can be concluded that: (i) the KR is both necessary and sufficient for ketoreduction to occur; (ii) this reduction always occurs at the C-9 position in the final polyketide backbone (counting from the carboxyl end of the chain); and (iii) while unreduced polyketides may undergo alternative cyclization patterns, in nascent polyketide chains that have undergone ketoreduction, the regiochemistry of the first cyclization is dictated by the position of the resulting hydroxyl, irrespective of how this cyclization occurs in the non-reduced product. In other words, the *tcm* PKS could be engineered to exhibit new cyclization specificity by including a ketoreductase.

A striking feature of RM20 (9) is the pattern of cyclizations following the first cyclization. Isolation of mutactin (6) from an *actVII* mutant suggested that the *actVII* product and its *tcm* homolog catalyze the cyclization of the second ring in the biosynthesis of actinorhodin (3) and tetracenomycin (5), respectively (Sherman, D.H. et al. *Tetrahedron* (1991) 47:6029; Summers, R.G. et al. *J. Bacteriol.* (1992) 174:1810). The cyclization pattern of RM20 (9) is different from that of 1 and tetracenomycin F1, despite the presence of the *actVII* gene on pRM20 (9). It therefore appears that the *act* cyclase cannot cyclize longer polyketide chains.

Unexpectedly, the strain containing the minimal *tcm* PKS alone (CH999/pSEK33) produced two polyketides, SEK15 (13) and SEK15b (16), as depicted in Figure 8, in

approximately equal quantities. Compounds (13) and (16) were also isolated from CH999/pSEK15, however, greater quantities of compound (13) were isolated than of compound (16).

5           SEK15b is a novel compound, the structure of which was elucidated through a combination of NMR spectroscopy, sodium [1,2- $^{13}\text{C}_2$ ] acetate feeding experiments and mass spectroscopy. Results from  $^1\text{H}$  and  $^{13}\text{C}$  NMR indicated that SEK15b consisted of an unreduced  
10 anthraquinone moiety and a pyrone moiety. Sodium [1,2- $^{13}\text{C}_2$ ]-acetate feeding experiments confirmed that the carbon chain of SEK15b was derived from 10 acetate units. The coupling constants calculated from the  $^{13}\text{C}$  NMR spectrum of the enriched SEK15b sample facilitated peak  
15 assignment. Fast atom bombardment (FAB) mass spectroscopy gave a molecular weight of 381 ( $M + \text{H}^+$ ), consistent with  $\text{C}_{20}\text{H}_{12}\text{O}_8$ . Deuterium exchange was used to confirm the presence of each hydroxyl in SEK15b.

          In order to identify the degrees of freedom  
20 available *in vivo* to a nascent polyketide chain for cyclizing in the absence of an active cyclase, polyketides produced by recombinant *S. coelicolor* CH999/pRM37 (McDaniel et al. (1993), *supra*) were analyzed. The biosynthetic enzymes encoded by pRM37 are  
25 the *tcm* ketosynthase/acyltransferase (KS/AT), the *tcm* chain length determining factor (CLF), the *tcm* acyl carrier protein (ACP), and the *act* ketoreductase (KR).

          Two novel compounds, RM20b (14) and RM20c (15) (Figure 8) were discovered in the culture medium of  
30 CH999/pRM37, which had previously yielded RM20 (9). The relative quantities of the three compounds recovered were 3:7:1 (RM20:RM20b:RM20c). The structures of (14) and (15) were elucidated through a combination of mass spectroscopy, NMR spectroscopy and isotope labeling  
35 experiments.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra suggested that RM20b

and RM20c were diastereomers, each containing a pyrone moiety. Optical rotations ( $[\alpha]_D^{20}$ ) were found to be +210.8° for RM20b (EtOH, 0.55%) and +78.0° for RM20c (EtOH, 0.33%). Sodium [1,2- $^{13}\text{C}_2$ ]-acetate feeding experiments confirmed that the carbon chain of RM20b (and by inference RM20c) was derived from 10 acetate units. Deuterium exchange studies were carried out in order to identify  $^1\text{H}$  NMR peaks corresponding to potential hydroxyl groups on both RM20b and RM20c. Proton coupling constants were calculated from the results of  $^1\text{H}$  NMR and one-dimensional decoupling experiments. In particular, the coupling pattern in the upfield region of the spectrum indicated a 5-proton spin system of two methylene groups surrounding a central carbinol methine proton. High resolution fast atom bombardment (FAB) mass spectroscopy gave molecular weights of (519.0056) ( $M = \text{Cs}^+$ ) for RM20b and 387.1070 ( $M + \text{H}^+$ ) for RM20c, which is consistent with  $\text{C}_{20}\text{H}_{18}\text{O}_8$  ( $M + \text{Cs}^+$ , 519.0056;  $M + \text{H}^+$ , 387.1080). Based on these data, structures (14) and (15) (Figure 8) were assigned to RM20b and RM20c, respectively.

Data from  $^1\text{H}$  and  $^{13}\text{C}$  NMR indicated that the coupling constants between H-9 and the geminal protons on C-8 were 12.1 or 12.2 and 2.5 or 2.2 Hz for RM20b or RM20c, respectively. The coupling constants between H-9 and the geminal protons on C-10 were 9.6 or 9.7 and 5.7 or 5.8 Hz for RM20b or RM20c, respectively. These values are typical of a  $J_{a,a}$  ( $J_{9a,8a}$  or  $J_{9a,10a}$ ) and  $J_{a,e}$  ( $J_{9a,8e}$  or  $J_{9a,10e}$ ) coupling pattern, and indicate an axial position for H-9 in both RM20b and RM20c. In contrast, the chemical shifts of the C-7 hydroxyls on the two molecules were 16.18 and 6.14 ppm for RM20b and RM20c, respectively. These values indicate a hydrogen bond between the C-7 hydroxyl and a suitably positioned acceptor atom in RM20b, but not in RM20c. The most

likely candidate acceptor atoms for such hydrogen bonding are the C-13 carbonyl oxygen in the conjugated pyrone ring system, or the bridge oxygen in the isolate pyrone ring. The former appears to be likely as it would be impossible to discriminate between (14) and (15) if the latter were the case. Furthermore, comparison of  $^{13}\text{C}$  NMR spectra of RM20b and RM20c revealed that the greatest differences between (14) and (15) were in the chemical shifts of the carbons that make up the conjugated pyrone ring (+5.9, -6.1, +8.9, -7.8 and +2.0 ppm for C-11, C-12, C-13, C-14 and C-15, respectively). Such a pattern of alternating upfield and downfield shifts can be explained by the fact that the C-7 hydroxyl is hydrogen-bonded to the C-13 carbonyl, since hydrogen bonding would be expected to reduce the electron density around C-11, C-13 and C-15, but increase the electron density around C-12 and C-14. To confirm the C-7/C-13 hydrogen bond assignment, the exchangeable protons RM20b and RM20c were replaced with deuterium (by incubating in the presence of  $\text{D}_2\text{O}$ ), and the samples were analyzed by  $^{13}\text{C}$  NMR. The C-13 peak in RM20b, but not RM20c, underwent an upfield shift (1.7 ppm), which can be explained by a weaker C-7/C-13 non-covalent bond in RM20b when hydrogen is replaced with deuterium. In order to form a hydrogen bond with the C-13 carbonyl, the C-7 hydroxyl of RM20b must occupy the equatorial position. Thus, it can be inferred that the C-7 and C-9 hydroxyls are on the same face (*syn*) of the conjugated ring system in the major isomer (RM20b), whereas they are on opposite sides (*anti*) in the minor isomer (RM20c).

No polyketide could be detected in CH999/pRM15, /pRM35, and /pRM36. Thus, only some ORF1-ORF2 combinations are functional. Since each subunit was functional in at least one recombinant synthase, protein expression/folding problems are unlikely to be the cause.

Instead, imperfect or inhibitory association between the different subunits of these enzyme complexes, or biosynthesis of (aborted) short chain products that are rapidly degraded, are plausible explanations.

5

B. Construction of hybrid PKSs including components from act and fren PKSs

*Streptomyces roseofulvus* produces both frenolicin B (7) (Iwai, Y. et al. *J. Antibiot.* (1978) 31:959) and nanaomycin A (8) (Tsuzuki, K. et al. *J. Antibiot.* (1986) 39:1343). A 10 kb DNA fragment (referred to as the *fren* locus hereafter) was cloned from a genomic library of *S. roseofulvus* (Bibb, M.J. et al. submitted) using DNA encoding the KS/AT and KR components of the act PKS of *S. coelicolor* A3(2) as a probe (Malpartida, F. et al. *Nature* (1987) 325:818). (See Figure 7 for structural representations.) DNA sequencing of the *fren* locus revealed the existence of (among others) genes with a high degree of identity to those encoding the act KS/AT, CLF, ACP, KR, and cyclase.

To produce the novel polyketides, the ORF1, 2 and 3 act genes present in pRM5 were replaced with the corresponding *fren* genes, as shown in Table 2. *S. coelicolor* CH999, constructed as described above, was transformed with these plasmids. (The genes encoding the act KR, and the act cyclase were also present on each of these genetic constructs.) Based on results from similar experiments with act and *tcm* PKSs, described above, it was expected that the act KR would be able to reduce the products of all functional recombinant PKSs, whereas the ability of the act cyclase to catalyze the second cyclization would depend upon the chain length of the product of the *fren* PKS.

The results summarized in Table 2 indicate that most of the transformants expressed functional PKSs, as



assayed by their ability to produce aromatic polyketides. Structural analysis of the major products revealed that the producer strains could be grouped into two categories: those that synthesized compound 1 (together with a smaller amount of its decarboxylated side-product (2), and those that synthesized a mixture of compounds 1, 10 and 11 in a roughly 1:2:2 ratio. (Small amounts of 2 were also found in all strains producing 1.) Compounds 1 and 2 had been observed before as natural products, and were the metabolites produced by a PKS consisting entirely of act subunits, as described in Example 3. Compounds 10 and 11 (designated RM18 and RM18b, respectively) are novel structures whose chemical synthesis or isolation as natural products has not been reported previously.

The structures of 10 and 11 were elucidated through a combination of mass spectroscopy, NMR spectroscopy, and isotope labeling experiments. The  $^1\text{H}$  and  $^{13}\text{C}$  spectral assignments are shown in Table 3, along with  $^{13}\text{C}$ - $^{13}\text{C}$  coupling constants for 10 obtained through sodium  $[1,2-^{13}\text{C}_2]$  acetate feeding experiments (described below). Unequivocal assignments for compound 10 were established with 1D nuclear Overhauser effect (NOE) and long range heteronuclear correlation (HETCOR) studies. Deuterium exchange confirmed the presence of hydroxyls at C-15 of compound 10 and C-13 of compound 11. Field desorption mass spectrometry (FD-MS) of 2 revealed a molecular weight of 282, consistent with  $\text{C}_{17}\text{H}_{14}\text{O}_4$  (282.2952).

Earlier studies showed that the polyketide backbone of 2 (Bartel, P.L. et al. *J. Bacteriol.* (1990) 172:4816) (and by inference, 1) is derived from iterative condensations of 8 acetate residues with a single ketoreduction at C-9. It may also be argued that nanaomycin (8) arises from an identical carbon chain

backbone. Therefore, it is very likely that nanaomycin is a product of the *fren* PKS genes in *S. roseofulvus*. Regiospecificity of the first cyclization leading to the formation of 1 is guided by the position of the  
5 ketoreduction, whereas that of the second cyclization is controlled by the *act* cyclase (Zhang, H.L. et al. *J. Org. Chem.* (1990) 55:1682).

In order to trace the carbon chain backbone of RM18 (10), *in vivo* feeding experiments using [1,2-<sup>13</sup>C<sub>2</sub>] acetate were performed on CH999/pRM18, followed by NMR  
10 analysis of labelled RM18 (10). The <sup>13</sup>C coupling data (summarized in Table 3) indicate that the polyketide backbone of RM18 (10) is derived from 9 acetate residues, followed by a terminal decarboxylation (the C-2 <sup>13</sup>C  
15 resonance appears as an enhanced singlet), which presumably occurs non-enzymatically. Furthermore, the absence of a hydroxyl group at the C-9 position suggests that a ketoreduction occurs at this carbon. Since these two features would be expected to occur in the putative  
20 frenolicin (7) backbone, the results suggest that, in addition to synthesizing nanaomycin, the *fren* PKS genes are responsible for the biosynthesis of frenolicin in *S. roseofulvus*. This appears to be the first unambiguous case of a PKS with relaxed chain length specificity.  
25 However, unlike the putative backbone of frenolicin, the C-17 carbonyl of RM18 (10) is not reduced. This could either reflect the absence from pRM18 of a specific ketoreductase, dehydratase, and an enoylreductase (present in the *fren* gene cluster in *S. roseofulvus*), or  
30 it could reflect a different origin for carbons 15-18 in frenolicin.

Regiospecificity of the first cyclization leading to the formation of RM18 (10) is guided by the position of the ketoreduction; however the second  
35 cyclization occurs differently from that in 7 or 1, and

is similar to the cyclization pattern observed in RM20 (9), a decaketide produced by the *tcm* PKS, as described above. Therefore, as in the case of RM20 (9), it could be argued that the act cyclase cannot catalyze the second cyclization of the RM18 precursor, and that its subsequent cyclizations, which presumably occur non-enzymatically, are dictated by temporal differences in release of different portions of the nascent polyketide chain into an aqueous environment. In view of the ability of CH999/pRM18 (and CH999/pRM34) to produce 1, one can rule out the possibility that the cyclase cannot associate with the *fren* PKS (KS/AT, CLF, and ACP). A more likely explanation is that the act cyclase cannot recognize substrates of altered chain lengths. This would also be consistent with the putative biosynthetic scheme for RM20 (9).

A comparison of the product profiles of the hybrid synthases reported in Table 2 with analogous hybrids between act and *tcm* PKS components (Table 1) support the hypothesis that the ORF2 product is the chain length determining factor (CLF). Preparation of compounds 9, 10 and 11 via cyclization of enzyme-bound ketides is schematically illustrated in Figure 8.

25

#### Example 5

#### Construction and Analysis of Modular Polyketide Synthases

Expression plasmids containing recombinant modular DEBS PKS genes were constructed by transferring DNA incrementally from a temperature-sensitive "donor" plasmid, i.e., a plasmid capable of replication at a first, permissive temperature and incapable of replication at a second, non-permissive temperature, to a "recipient" shuttle vector via a double recombination event, as depicted in Figure 10. pCK7 (Figure 11), a shuttle plasmid containing the complete *eryA* genes, which

were originally cloned from pS1 (Tuan et al. (1990) *Gene* 90:21), was constructed as follows. A 25.6 kb *SphI* fragment from pS1 was inserted into the *SphI* site of pMAK705 (Hamilton et al. (1989) *J. Bacteriol.* 171:4617) to give pCK6 ( $\text{Cm}^R$ ), a donor plasmid containing *eryAII*, *eryAIII*, and the 3' end of *eryAI*. Replication of this temperature-sensitive pSC101 derivative occurs at 30°C but is arrested at 44°C. The recipient plasmid, pCK5 ( $\text{Ap}^R$ ,  $\text{Tc}^R$ ), includes a 12.2kb *eryA* fragment from the *eryAI* start codon (Caffrey et al. (1992) *FEBS Lett.* 304:225) to the *XcmI* site near the beginning of *eryAII*, a 1.4 kb *EcoRI* - *BsmI* pBR322 fragment encoding the tetracycline resistance gene (*Tc*), and a 4.0 kb *NotI* - *EcoRI* fragment from the end of *eryAIII*. *PacI*, *NdeI*, and *XbaI* ribosome binding sites were engineered at the *eryAI* start codon in pCK5. pCK5 is a derivative of pRM5 (McDaniel et al. (1993), *supra*). The 5' and 3' regions of homology (Figure 10, striped and unshaded areas) are 4.1 kb and 4.0 kb, respectively. MC1061 *E. coli* was transformed (see, Sambrook et al., *supra*) with pCK5 and pCK6 and subjected to carbenicillin and chloramphenicol selection at 30°C. Colonies harboring both plasmids ( $\text{Ap}^R$ ,  $\text{Cm}^R$ ) were then restreaked at 44°C on carbenicillin and chloramphenicol plates. Only cointegrates formed by a single recombination event between the two plasmids were viable. Surviving colonies were propagated at 30°C under carbenicillin selection, forcing the resolution of the cointegrates via a second recombination event. To enrich for pCK7 recombinants, colonies were restreaked again on carbenicillin plates at 44°C. Approximately 20% of the resulting colonies displayed the desired phenotype ( $\text{Ap}^R$ ,  $\text{Tc}^S$ ,  $\text{Cm}^S$ ). The final pCK7 candidates were thoroughly checked via restriction mapping. A control plasmid, pCK7f, which contains a frameshift error in *eryAI*, was constructed in a similar manner. pCK7 and pCK7f were

transformed into *E. coli* ET12567 (MacNeil (1988) *J. Bacteriol.* 170:5607) to generate unmethylated plasmid DNA and subsequently moved into *Streptomyces coelicolor* CH999 using standard protocols (Hopwood et al. (1985) *Genetic*  
5 *manipulation of Streptomyces. A laboratory manual.* The John Innes Foundation: Norwich).

Upon growth of CH999/pCK7 on R2YE medium, the organism produced abundant quantities of two polyketides (Figure X). The addition of propionate (300 mg/L) to the  
10 growth medium resulted in approximately a two-fold increase in yield of polyketide product. Proton and  $^{13}\text{C}$  NMR spectroscopy, in conjunction with propionic-1- $^{13}\text{C}$  acid feeding experiments, confirmed the major product as 6dEB (17) (> 40 mg/L). The minor product was identified  
15 as 8,8a-deoxyoleandolide (18) (> 10 mg/L), which apparently originates from an acetate starter unit instead of propionate in the 6dEB biosynthetic pathway.  $^{13}\text{C}_2$  sodium acetate feeding experiments confirmed the incorporation of acetate into (18). Three high molecular  
20 weight proteins (>200 kDa), presumably DEBS1, DEBS2, and DEBS3 (Caffrey et al. (1992) *FEBS Lett.* 304:225), were also observed in crude extracts of CH999/pCK7 via SDS-polyacrylamide gel electrophoresis. No polyketide  
products were observed from CH999/pCK7f.

25

---

Thus, novel polyketides, as well as methods for recombinantly producing the polyketides, are disclosed.  
30 Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

35

TABLE 1

	Plasmid	ORF1 (KS/AT)	ORF2 (CLDF)	ORF3 (ACP)	Major Product(s)	Backbone Carbon Length
5	pRM5	<i>act</i>	<i>act</i>	<i>act</i>	1,2	16
	pRM7	<i>gra</i>	<i>act</i>	<i>act</i>	1,2	16
	pRM12	<i>act</i>	<i>gra</i>	<i>act</i>	1,2	16
	pRM22	<i>act</i>	<i>act</i>	<i>gra</i>	1,2	16
10	pRM10	<i>tcm</i>	<i>act</i>	<i>act</i>	1,2	16
	pRM15	<i>act</i>	<i>tcm</i>	<i>act</i>	NP	--
	pRM20	<i>tcm</i>	<i>tcm</i>	<i>act</i>	9	20
	pRM25	<i>act</i>	<i>act</i>	<i>tcm</i>	1,2	16
15	pRM35	<i>tcm</i>	<i>act</i>	<i>tcm</i>	NP	--
	pRM36	<i>act</i>	<i>tcm</i>	<i>tcm</i>	NP	--
	pRM37	<i>tcm</i>	<i>tcm</i>	<i>tcm</i>	9,14,15	20
	pSEK15	<i>tcm</i>	<i>tcm</i>	<i>tcm</i>	13,16	20
20	pSEK33	<i>tcm</i>	<i>tcm</i>	<i>act</i>	13,16	20

TABLE 2

	Plasmid	ORF1 (KS/AT)	ORF2 (CLDF)	ORF3 (ACP)	Major Product(s)
25	pRM5	<i>act</i>	<i>act</i>	<i>act</i>	1,2
	pRM8	<i>fren</i>	<i>act</i>	<i>act</i>	1,2
	pRM13	<i>act</i>	<i>fren</i>	<i>act</i>	NP
	pRM23	<i>act</i>	<i>act</i>	<i>fren</i>	1,2
30	pRM18	<i>fren</i>	<i>fren</i>	<i>act</i>	1,2,10,11
	pRM32	<i>fren</i>	<i>act</i>	<i>fren</i>	NP
	pRM33	<i>act</i>	<i>fren</i>	<i>fren</i>	NP
	pRM34	<i>fren</i>	<i>fren</i>	<i>fren</i>	0001,2,10, 11
35					

TABLE 3

<sup>1</sup>H(400 MHz) and <sup>13</sup>C(100 MHz) NMR data from RM18(10) and RM18b(11)

carbon <sup>α</sup>	RM18			RM18b		
	<sup>13</sup> Cδ(ppm)	<sup>1</sup> J <sub>CC</sub> (Hz)	<sup>1</sup> Hδ(ppm) (m, <sup>1</sup> J <sub>HH</sub> (Hz), area))	carbon <sup>α</sup>	<sup>13</sup> Cδ(ppm)	<sup>1</sup> Hδ(ppm) (m, <sup>1</sup> J <sub>HH</sub> (Hz), area))
2	29.6	NC <sup>b</sup>	2.2(s, 3H)			
3	203.7	37.7				
4	47.0	36.9	3.6(s, 2H)	2	18.8	2.1(s, 3H)
5	149.6	77.2		3	152.3	
6	106.7	77.4	6.2(s, 1H)	4	104.0	6.1(s, 1H)
7	129.1	61.9		5	130.0	
8	114.4	62.1	6.7(d, 7.2, 1H)	6	113.5	6.7(d, 7.0)
9	130.1	58.9	7.3(dd, 8.4, 7.4, 1H)	7	130.1	7.3(dd, 7.1, 8.7, 1H)
10	120.6	59.2	7.6(d, 8.9, 1H)	8	120.1	7.6(d, 8.6, 1H)
11	132.7	56.0		9	132.8	
12	116.7	55.7		10	116.6	
13	155.6	74.7		11	155.9	
14	98.4	74.9		12	98.2	
15	158.8	69.6	6.4(s, 1H)	13	159.1	6.4(s, 1H)
16	113.6	69.3	11.2(s, 1OH)	14	113.8	11.2(s, 1OH)
17	201.7	41.9		15	201.7	
18	32.4	41.7	2.5(s, 3H)	16	32.4	2.5(s, 3H)

<sup>α</sup> carbons are labelled according to their number in the polyketide backbone  
<sup>b</sup> NC, not coupled

Table 4.  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for SEK4 (12) and SEK15 (13)<sup>a</sup>

carbon <sup>b</sup>	SEK4 (12)			carbon	SEK15 (13)		
	$^{13}\text{C}\delta(\text{ppm})$	$J_{\text{CC}}(\text{Hz})$	$^1\text{H}\delta(\text{ppm})$		$^{13}\text{C}\delta(\text{ppm})$	$J_{\text{CC}}(\text{Hz})$	$^1\text{H}\delta(\text{ppm})$
1	165.4	78.8	11.60(s, 1OH)	1	164.0	79.1	12.20(s, 1OH)
2	88.2	79.8	6.26(d, J=2.28 Hz, 1H)	2	88.2	79.4	6.20(d, J=1.88 Hz, 1H)
3	170.5	55.3		3	172.8	57.9	
4	111.3	61.3	6.33(d, J=2.24 Hz, 1H)	4	101.8	53.9	6.20(d, J=1.88 Hz, 1H)
5	163.8	51.0		5	163.1	50.4	
6	37.6	50.8	4.07(d, J=15.7 Hz, 1H) 4.16(d, J=16.0 Hz, 1H)	6	36.7	50.8	1.90(s, 2H)
7	138.6	60.7		7	135.4	60.7	
8	102.9	60.9	5.66(d, J=1.6 Hz, 1H)	8	109.1	61.7	5.66(s, 1H)
9	161.9	71.9	10.50(s, 1OH)	9	159.8	66.2	
10	100.6	70.9	5.19(d, J=1.96 Hz, 1H)	10	101.6	66.5	5.08(s, 1H)
11	162.9	60.8		11	157.4	67.3	
12	112.9	61.6		12	121.1	67.6	
13	191.1	39.1		13	200.3	58.1	
14	49.3	39.9	2.54(d, J=15.9 Hz, 1H) 4.92(d, J=16.0 Hz, 1H)	14	117.2	58.6	
15	99.6	46.6	6.90(s, 1OH)	15	163.6	68.5	
16	27.5	46.8	1.56(s, 3H)	16	100.6	68.0	6.08(s, 1H)
				17	162.2	62.6	
				18	111.0	62.0	6.12(s, 1H)
				19	141.9	43.3	
				20	21.1	42.7	1.86(s, 3H)

a.  $^1\text{H}$  and  $^{13}\text{C}$  NMR's were recorded in DMSO- $d_6$  (400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$ )

b. carbons are labelled according to their number in the polyketide backbone



Claims

1. A genetically engineered cell which  
5 expresses a polyketide synthase (PKS) gene cluster in its native, nontransformed state, said genetically engineered cell substantially lacking the entire native PKS gene cluster.
- 10 2. The genetically engineered cell of claim 1, wherein the cell is a procaryotic cell.
3. The genetically engineered cell of claim 2, wherein the cell is an actinomycete.
- 15 4. The genetically engineered cell of claim 3, wherein the cell is an actinomycete of the genus *Streptomyces*.
- 20 5. The genetically engineered cell of claim 4, wherein the cell is *Streptomyces coelicolor*.
6. The genetically engineered cell of claim 5, wherein the cell substantially lacks the entire native  
25 actinorhodin PKS gene cluster.
7. The genetically engineered cell of claim 6, wherein the cell is equivalent to strain CH999.
- 30 8. The genetically engineered cell of any of claims 1-7, wherein the cell comprises:  
(a) a replacement PKS gene cluster which encodes a PKS capable of catalyzing the synthesis of a polyketide; and

35

(b) one or more control sequences operatively linked to said PKS gene cluster, whereby the genes in said gene cluster can be transcribed and translated in the genetically engineered cell,

5           with the proviso that when the replacement PKS gene cluster comprises an entire PKS gene set, at least one of the PKS genes or control elements is heterologous to the cell.

10           9. The genetically engineered cell of claim 8, wherein the replacement PKS gene cluster comprises a first gene encoding a PKS ketosynthase and a PKS acyltransferase active site (KS/AT), a second gene  
15           encoding a PKS chain length determining factor (CLF), and a third gene encoding a PKS acyl carrier protein (ACP).

            10. The genetically engineered cell of claim 9, wherein the replacement PKS gene cluster further  
20           comprises a fourth gene encoding a PKS ketoreductase (KR).

            11. The genetically engineered cell of claim 10, wherein the replacement PKS gene cluster further  
25           comprises a fifth gene encoding a PKS cyclase (CYC).

            12. The genetically engineered cell of claim 11, wherein the replacement PKS gene cluster further  
            comprises a sixth gene encoding a PKS dehydratase.

30           13. The genetically engineered cell of claim 9, wherein the PKS KS/AT gene is derived from a PKS KS/AT gene selected from the group consisting of an actinorhodin KS/AT gene, a granaticin KS/AT gene, a tetracenomycin KS/AT gene, a frenolicin B KS/AT gene, an  
35           oxytetracycline KS/AT gene, and a griseusin KS/AT gene.

14. The genetically engineered cell of claim 9, wherein the PKS CLF gene is derived from a PKS CLF gene selected from the group consisting of an actinorhodin CLF gene, a granaticin CLF gene, a tetracenomycin CLF gene, a frenolicin B CLF gene, an oxytetracycline CLF gene, and a griseusin CLF gene.

15. The genetically engineered cell of claim 9, wherein the PKS ACP gene is derived from a PKS ACP gene selected from the group consisting of an actinorhodin ACP gene, a granaticin ACP gene, a tetracenomycin ACP gene, a frenolicin B ACP gene, an oxytetracycline ACP gene, and a griseusin ACP gene.

16. The genetically engineered cell of claim 10, wherein the PKS KR gene is derived from a PKS KR gene selected from the group consisting of an actinorhodin KR gene, a granaticin KR gene, a tetracenomycin KR gene, a frenolicin B KR gene, an oxytetracycline KR gene, and a griseusin KR gene.

17. The genetically engineered cell of claim 9, wherein each of said first, second and third genes are contained in separate expression cassettes.

18. The genetically engineered cell of claim 17, wherein the separate expression cassettes are present in a single vector.

19. The genetically engineered cell of claim 17, wherein the separate expression cassettes are present in two or more vectors.

20. The genetically engineered cell of claim 8, wherein the replacement gene cluster comprises a first.

gene encoding a PKS acyltransferase (AT), a second gene encoding a PKS ketoacyl carrier protein synthase (KS), a third gene encoding a PKS acyl carrier protein (ACP), a fourth gene encoding a PKS ketoreductase (KR), a fifth  
5 gene encoding a PKS dehydratase (DH), a sixth gene encoding a PKS enoyl reductase (ER), and a seventh gene encoding a thioesterase (TE).

21. The genetically engineered cell of claim  
10 20, wherein the genes are derived from the 6-deoxyerythronolide B synthase gene cluster.

22. A method for producing a recombinant polyketide comprising:

- 15 (a) providing a population of cells according to any of claims 1-21; and  
(b) culturing the population of cells under conditions whereby the replacement PKS gene cluster present in the cells, is expressed.

20

23. A method for producing a recombinant polyketide comprising:

- a. inserting a first portion of a replacement PKS gene cluster into a donor plasmid and inserting a  
25 second portion of a replacement PKS gene cluster into a recipient plasmid, wherein the first and second portions collectively encode a complete replacement PKS gene cluster, and further wherein:  
i. the donor plasmid expresses a gene  
30 which encodes a first selection marker and is capable of replication at a first, permissive temperature and incapable of replication at a second, non-permissive temperature;  
ii. the recipient plasmid expresses a  
35 gene which encodes a second selection marker; and

iii. the donor plasmid comprises regions of DNA complementary to regions of DNA in the recipient plasmid, such that homologous recombination can occur between the first portion of the replacement PKS gene cluster and the second portion of the replacement gene cluster, whereby a complete replacement gene cluster can be generated;

b. transforming the donor plasmid and the recipient plasmid into a host cell and culturing the transformed host cell at the first, permissive temperature and under conditions which allow the growth of host cells which express the first and/or the second selection markers, to generate a first population of cells;

c. culturing the first population of cells at the second, non-permissive temperature and under conditions which allow the growth of cells which express the first and/or the second selection markers, to generate a second population of cells which includes host cells which contain a recombinant plasmid comprising a complete PKS replacement gene cluster;

d. transferring the recombinant plasmid from the second population of cells into the genetically engineered cell of claim 1 to generate transformed genetically engineered cells; and

e. culturing the transformed genetically engineered cells under conditions whereby the replacement PKS gene cluster present in the cells is expressed.

24. The method of claim 23, wherein the method further comprises after step (c) culturing the second population of cells at the first, permissive temperature and under conditions which allow the growth of cells which express the first selection marker.

25. The method of claim 23, wherein the first and second portions of the replacement PKS gene cluster are derived from the 6-deoxyerythronolide B synthase gene cluster.

5

26. A polyketide produced by the method of any of claims 1-25.

27. A recombinant vector comprising:

10

(a) a DNA sequence comprising a modular replacement PKS gene cluster; and

(b) control elements that are operably linked to said DNA sequence whereby said DNA sequence can be transcribed and translated in a host cell and at least one of said control elements is heterologous to said nucleotide sequence.

15

28. The recombinant vector of claim 90, wherein the replacement gene cluster is the 6-deoxyerythronolide B synthase gene cluster.

20

29. The plasmid pCK7.

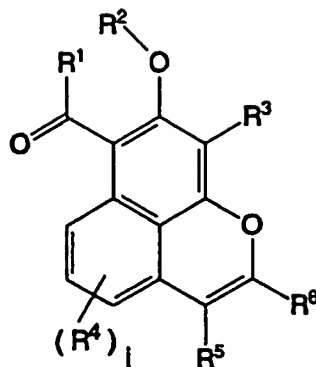
30. A host cell transformed with the vector of any of claims 27-30.

25

31. A polyketide compound having the structural formula

30

35



wherein:

R¹ is selected from the group consisting of hydrogen and lower alkyl and R² is selected from the group consisting of hydrogen, lower alkyl and lower alkyl ester, or wherein R¹ and R² together form a lower alkylene bridge optionally substituted with one to four hydroxyl or lower alkyl groups;

R³ and R⁵ are independently selected from the group consisting of hydrogen, halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro;

R⁴ is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro;

R⁶ is selected from the group consisting of hydrogen, lower alkyl, and -CHR⁷-(CO)R⁸ where R⁷ and R⁸ are independently selected from the group consisting of hydrogen and lower alkyl; and

i is 1, 2 or 3.

32. The compound of claim 31, wherein:

R<sup>1</sup> is lower alkyl;

R<sup>2</sup>, R<sup>3</sup> and R<sup>5</sup> are hydrogen;

R<sup>6</sup> is -CHR<sup>7</sup>-(CO)-R<sup>8</sup>; and

i is 0.

5

33. The compound of claim 32, wherein R<sup>1</sup> is methyl and R<sup>6</sup> is -CH<sub>2</sub>-(CO)-CH<sub>3</sub>.

10

34. The compound of claim 31, wherein:

R<sup>1</sup> and R<sup>6</sup> are lower alkyl;

R<sup>2</sup>, R<sup>3</sup> and R<sup>5</sup> are hydrogen; and

i is 0.

15

35. The compound of claim 34, wherein R<sup>1</sup> and R<sup>6</sup> are methyl.

20

36. The compound of claim 31, wherein R<sup>1</sup> and R<sup>2</sup> are linked together to form a lower alkylene bridge - CHR<sup>9</sup>-CHR<sup>10</sup> wherein R<sup>9</sup> and R<sup>10</sup> are independently selected from the group consisting of hydrogen, hydroxyl and lower alkyl.

25

37. The compound of claim 31, wherein R<sup>1</sup> and R<sup>2</sup> are linked together to form a lower alkylene bridge - CH<sub>2</sub>-CHOH-.

30

38. The compound of claim 37, wherein:

R<sup>3</sup> and R<sup>5</sup> are hydrogen; and

i is 0.

35

39. The compound of claim 38, wherein R<sup>6</sup> is -CHR<sup>7</sup>-(CO)-R<sup>8</sup> where R<sup>8</sup> is hydrogen or lower alkyl.

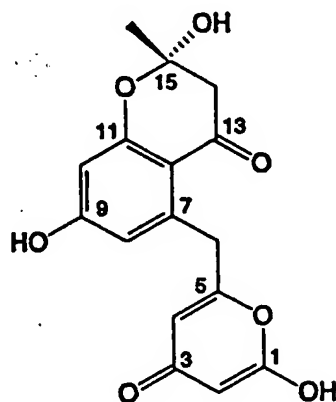


40. The compound of claim 39, wherein R<sup>6</sup> is  
-CH<sub>2</sub>-(CO)-CH<sub>3</sub>.

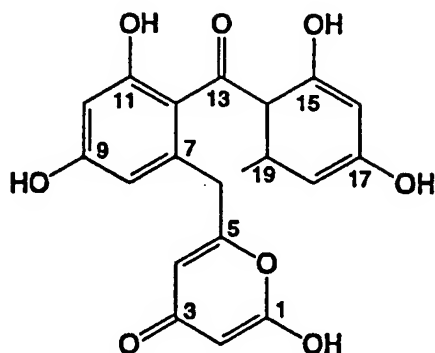
41. The compound of claim 38, wherein R<sup>6</sup> is  
5 lower alkyl.

42. The compound of claim 41 wherein R<sup>6</sup> is  
methyl.

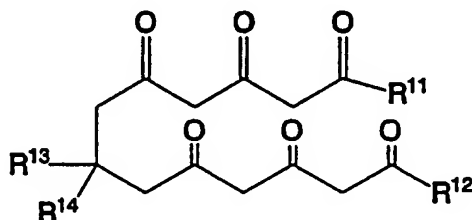
10 43. A polyketide compound having the  
structural formula



44. A polyketide compound having the structural formula



45. A polyketide compound formed by catalytic cyclization of an enzyme-bound ketide having the structure



wherein:

$R^{11}$  is selected from the group consisting of methyl,  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $-\text{CH}_2(\text{CO})\text{CH}_2(\text{CO})\text{CH}_3$ ;

$R^{12}$  is selected from the group consisting of  $-\text{S-E}$  and  $-\text{CH}_2(\text{CO})-\text{S-E}$ , wherein E represents a polyketide synthase produced by the genetically engineered cell of claim 8; and

one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl, or  $R^{13}$  and  $R^{14}$  together represent carbonyl.

46. The compound of claim 45, wherein  $R^{11}$  is methyl and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ .

47. The compound of claim 45, wherein  $R^{11}$  is  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{S}-\text{E}$ .

48. The compound of claim 45, wherein  $R^{11}$  is  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ .

49. The compound of claim 45, wherein  $R^{11}$  is  $-\text{CH}_2(\text{CO})\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ .

50. The compound of claim 46, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.

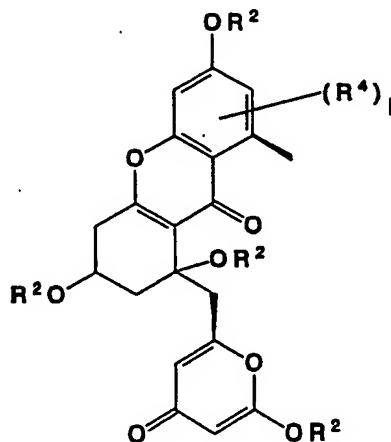
51. The compound of claim 47, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.

52. The compound of claim 48, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.

53. The compound of claim 49, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.

54. The compound of claim 50, wherein  $R^{13}$  and  $R^{14}$  together form a carboxyl moiety.

55. A polyketide compound having the structural formula



wherein;

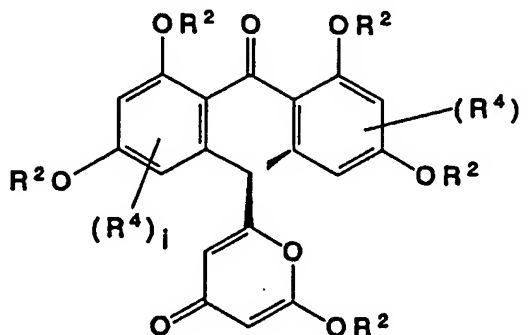
the  $R^2$  moieties are independently selected from the group consisting of hydrogen, lower alkyl and lower alkyl esters;

$R^4$  is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro; and

$i$  is 0, 1 or 2.

56. The compound of claim 55, wherein  $R^2$  is hydrogen and  $i$  is 0.

57. A polyketide compound having the structural formula



15 wherein:

the  $R^2$  moieties are independently selected from the group consisting of hydrogen, lower alkyl and lower alkyl esters;

20  $R^4$  is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro; and

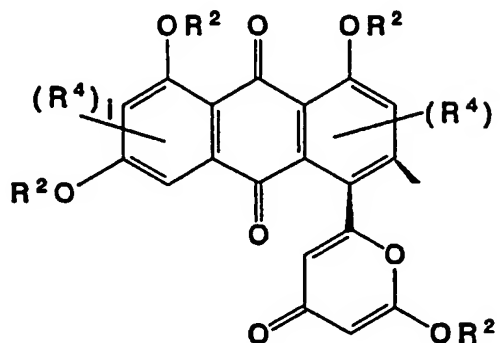
$i$  is 0, 1 or 2.

58. The compound of claim 57, wherein  $R^2$  is  
25 hydrogen and  $i$  is 0.

30

35

59. A polyketide compound having the structural formula



15 wherein:

the R² moieties are independently selected from the group consisting of hydrogen, lower alkyl and lower alkyl esters;

20 R⁴ is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro; and  
i is 0, 1 or 2.

60. The compound of claim 59, wherein R² is  
25 hydrogen and i is 0.

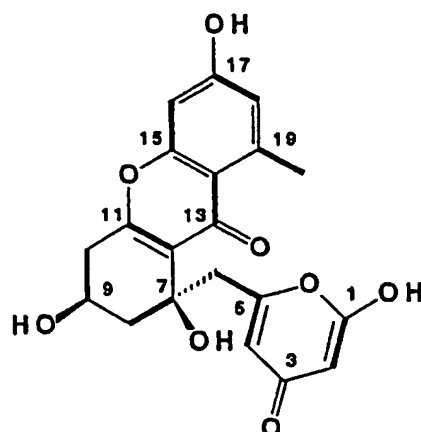
30

35

61. A polyketide compound having the structural formula

5

10

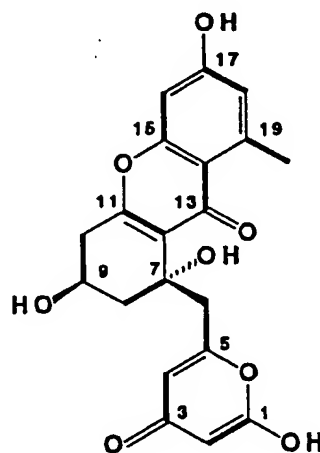


15

62. A polyketide compound having the structural formula

20

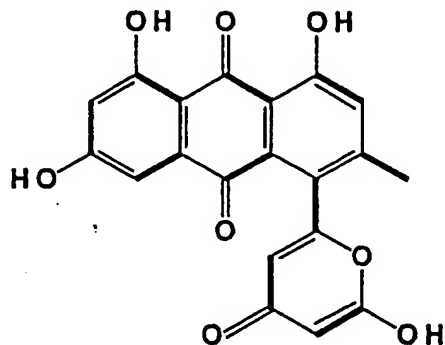
25



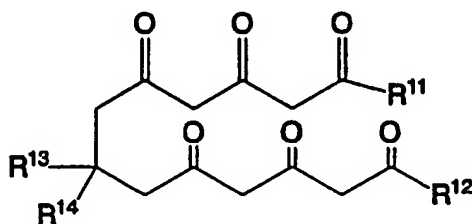
30

35

63. A polyketide compound having the structural formula



64. A method for producing an aromatic polyketide, comprising effecting cyclization of an enzyme-bound ketide having the structure



wherein:

$R^{11}$  is selected from the group consisting of methyl,  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $-\text{CH}_2(\text{CO})\text{CH}_2(\text{CO})\text{CH}_3$ ;

$R^{12}$  is selected from the group consisting of  $-\text{S}-\text{E}$  and  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ , wherein E represents a polyketide synthase produced by the genetically engineered cell of claim 8; and



one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl, or  $R^{13}$  and  $R^{14}$  together represent carbonyl, wherein cyclization is induced by said polyketide synthase.

5

65. The method of claim 64, wherein  $R^{11}$  is methyl and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ .

66. The method of claim 64, wherein  $R^{11}$  is  
10  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{S}-\text{E}$ .

67. The method of claim 64, wherein  $R^{11}$  is  $-\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ .

68. The method of claim 64, wherein  $R^{11}$  is  
15  $-\text{CH}_2(\text{CO})\text{CH}_2(\text{CO})\text{CH}_3$  and  $R^{12}$  is  $-\text{CH}_2(\text{CO})-\text{S}-\text{E}$ .

69. The method of claim 65, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.  
20

70. The method of claim 66, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.

71. The method of claim 67, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.  
25

72. The method of claim 68, wherein one of  $R^{13}$  and  $R^{14}$  is hydrogen and the other is hydroxyl.

73. The method of claim 68, wherein  $R^{13}$  and  $R^{14}$  together form a carboxyl moiety.  
30

35

## AMENDED CLAIMS

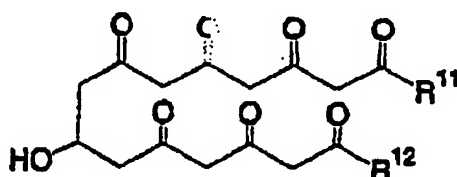
[received by the International Bureau on 27 February 1995 (27.02.95); original claims 43-44, 49-54 and 68-73 cancelled; original claims 45-48 and 64-67 replaced by new claims 43-46 and 47-50, respectively; original claims 55-63 renumbered into new claims 51-59; remaining claims unchanged (7 pages)]

40. The compound of claim 39, wherein  $R^6$  is  $-\text{CH}_2-(\text{CO})-\text{CH}_3$ .

41. The compound of claim 38, wherein  $R^6$  is lower alkyl.

42. The compound of claim 41 wherein  $R^6$  is methyl.

43. A polyketide compound formed by catalytic cyclization of an enzyme-bound ketide having the structure



wherein:

$R^{11}$  is selected from the group consisting of methyl and  $-\text{COCH}_3$ ;

$R^{12}$  is selected from the group consisting of  $-\text{S-E}$  and  $-(\text{CO})-\text{S-E}$ ; and

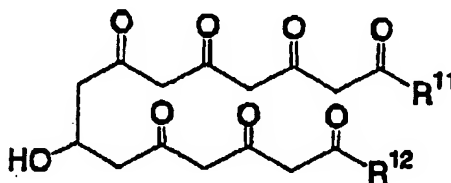
E represents a polyketide synthase produced by the genetically engineered cell of claim 8.

44. The compound of claim 43, wherein  $R^{11}$  is methyl and  $R^{12}$  is  $-\text{S-E}$ .

45. The compound of claim 43, wherein  $R^{11}$  is methyl and  $R^{12}$  is  $-(\text{CO})-\text{S-E}$ .

46. The compound of claim 43, wherein  $R^{11}$  is  $-\text{COCH}_3$  and  $R^{12}$  is  $-(\text{CO})-\text{S-E}$ .

47. A method for producing an aromatic polyketide, comprising effecting cyclization of an enzyme-bound ketide having the structure



wherein:

$R^{11}$  is selected from the group consisting of methyl and  $-\text{COCH}_3$ ;

$R^{12}$  is selected from the group consisting of  
15  $-\text{S}-\text{E}$  and  $-(\text{CO})-\text{S}-\text{E}$ ; and

$\text{E}$  represents a polyketide synthase produced by the genetically engineered cell of claim 8, wherein cyclization is induced by said polyketide synthase.

20 48. The method of claim 47, wherein  $R^{11}$  is methyl and  $R^{12}$  is  $-\text{S}-\text{E}$ .

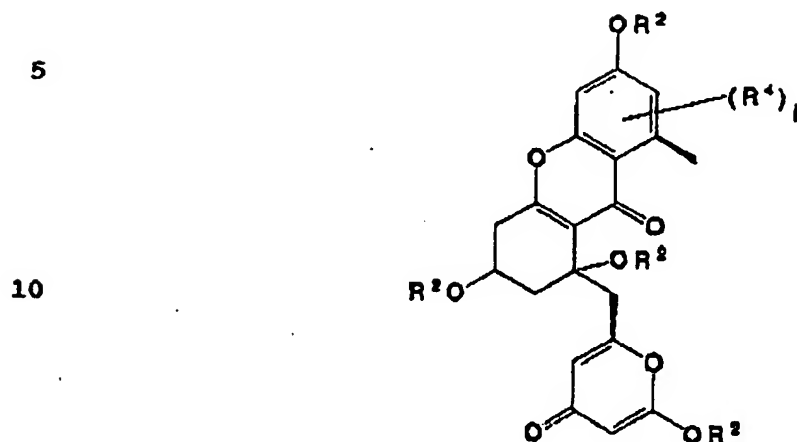
49. The method of claim 47, wherein  $R^{11}$  is methyl and  $R^{12}$  is  $-(\text{CO})-\text{S}-\text{E}$ .

25 50. The method of claim 47, wherein  $R^{11}$  is  $-\text{COCH}_3$  and  $R^{12}$  is  $-(\text{CO})-\text{S}-\text{E}$ .

30

35

51. A polyketide compound having the structural formula



wherein;

the  $R^2$  moieties are independently selected from the group consisting of hydrogen, lower alkyl and lower alkyl esters;

$R^4$  is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro; and

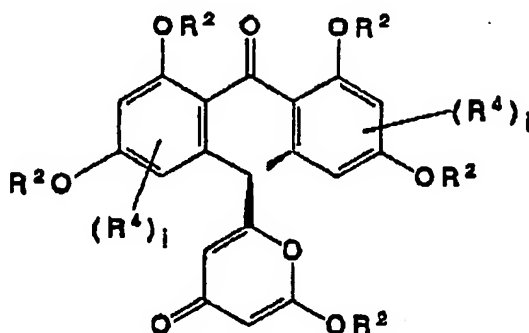
$i$  is 0, 1 or 2.

52. The compound of claim 51, wherein  $R^2$  is hydrogen and  $i$  is 0.

30

35

53. A polyketide compound having the structural formula



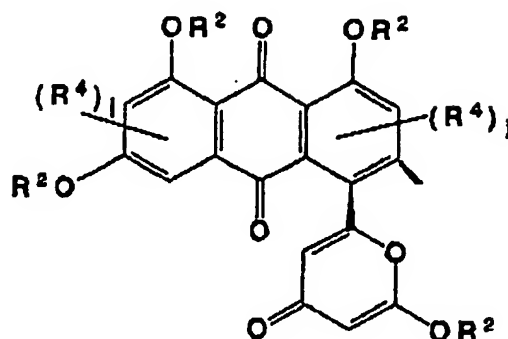
wherein:

15 the  $R^2$  moieties are independently selected from the group consisting of hydrogen, lower alkyl and lower alkyl esters;

$R^4$  is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl  
20 mono- or di-substituted amino and nitro; and  
 $i$  is 0, 1 or 2.

54. The compound of claim 53, wherein  $R^2$  is hydrogen and  $i$  is 0.

55. A polyketide compound having the structural formula



wherein:

the R<sup>2</sup> moieties are independently selected from the group consisting of hydrogen, lower alkyl and lower alkyl esters;

R<sup>4</sup> is selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkyl mono- or di-substituted amino and nitro; and

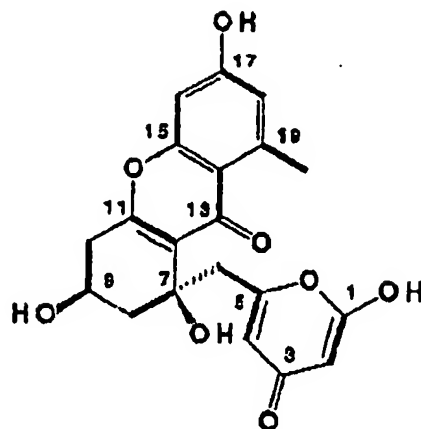
i is 0, 1 or 2.

56. The compound of claim 55, wherein R<sup>2</sup> is hydrogen and i is 0.

57. A polyketide compound having the structural formula

5

10

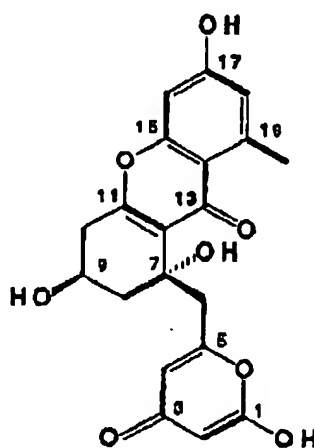


15

58. A polyketide compound having the structural formula

20

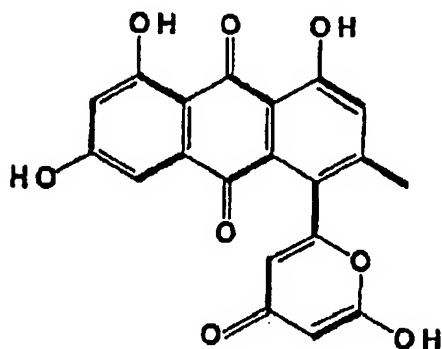
25



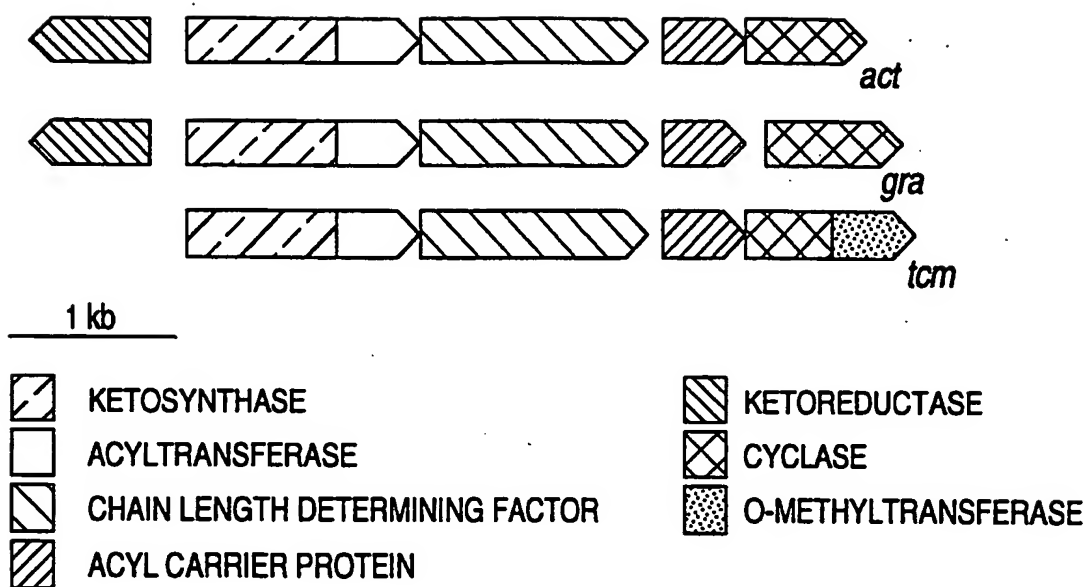
30

35

59. A polyketide compound having the structural formula





**FIG. 1**

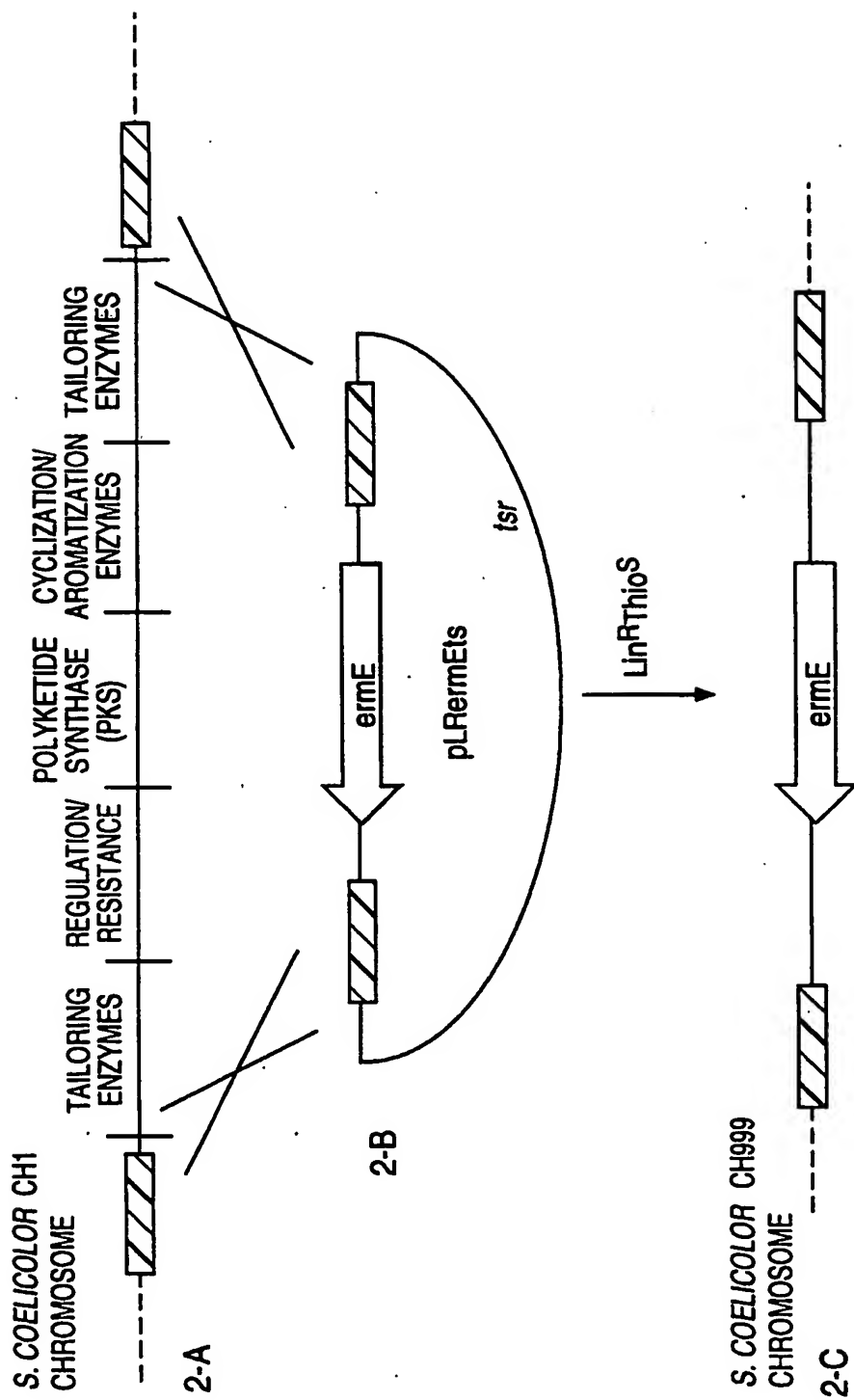
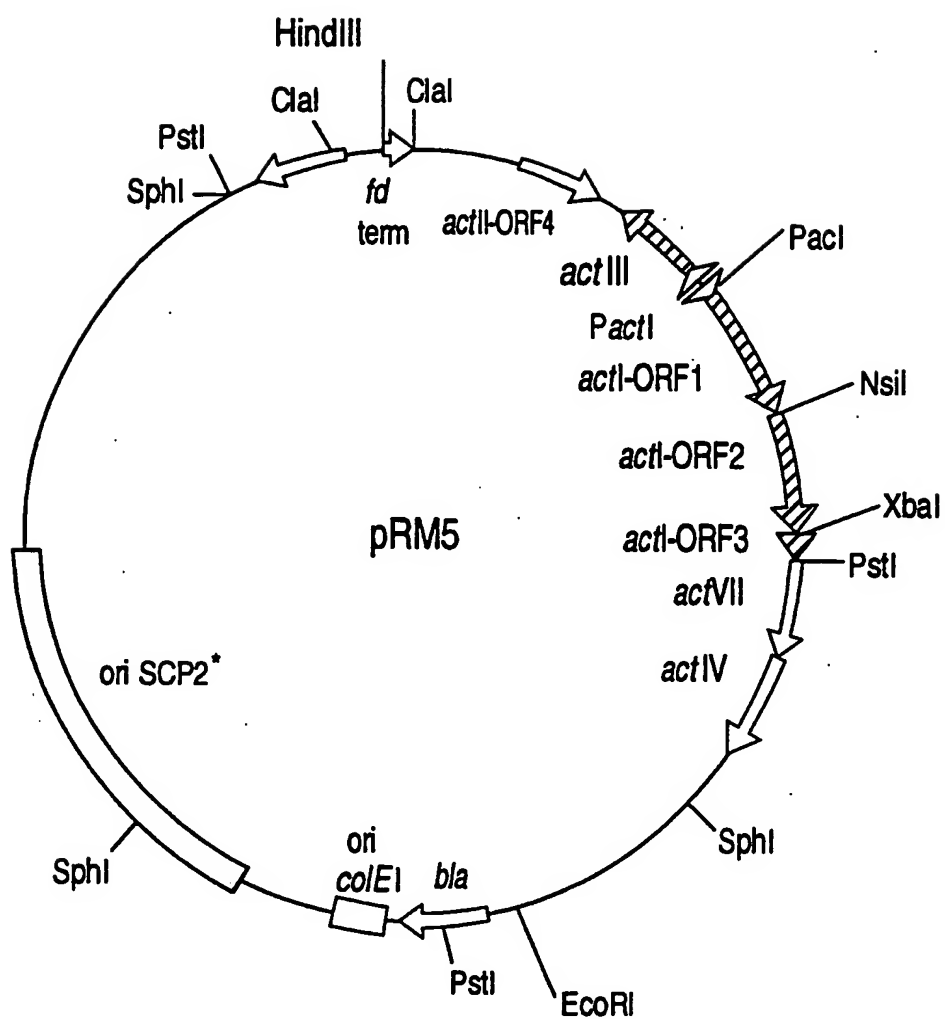
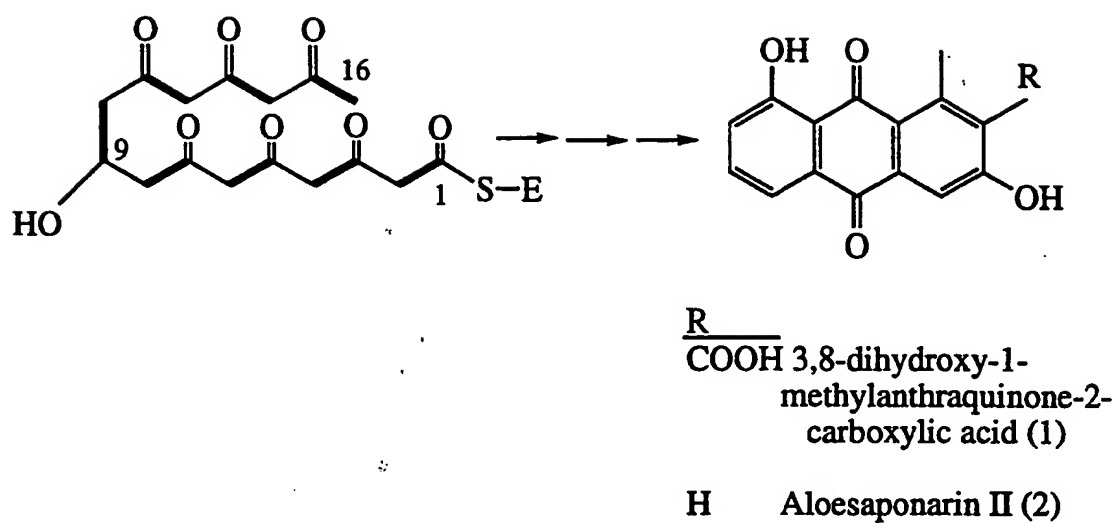
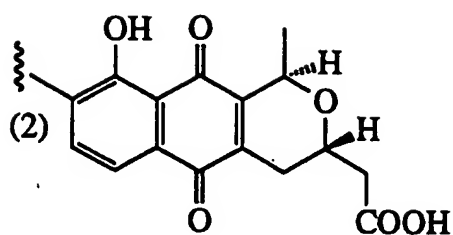


FIG. 2

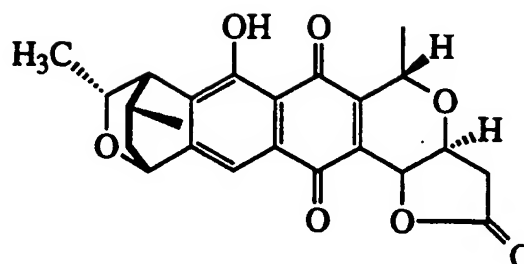
**FIG. 3**



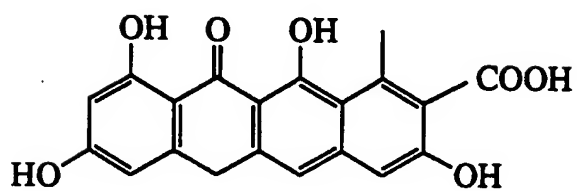
**FIG. 4**



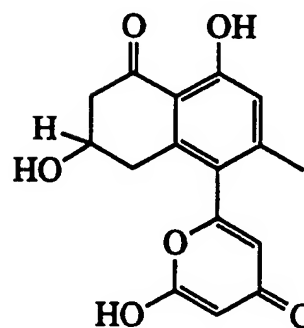
ACTINORHODIN (3)



GRANATICIN (4)



TETRACENOMYCIN F1 (5)



MUTACTIN (6)

FIG. 5

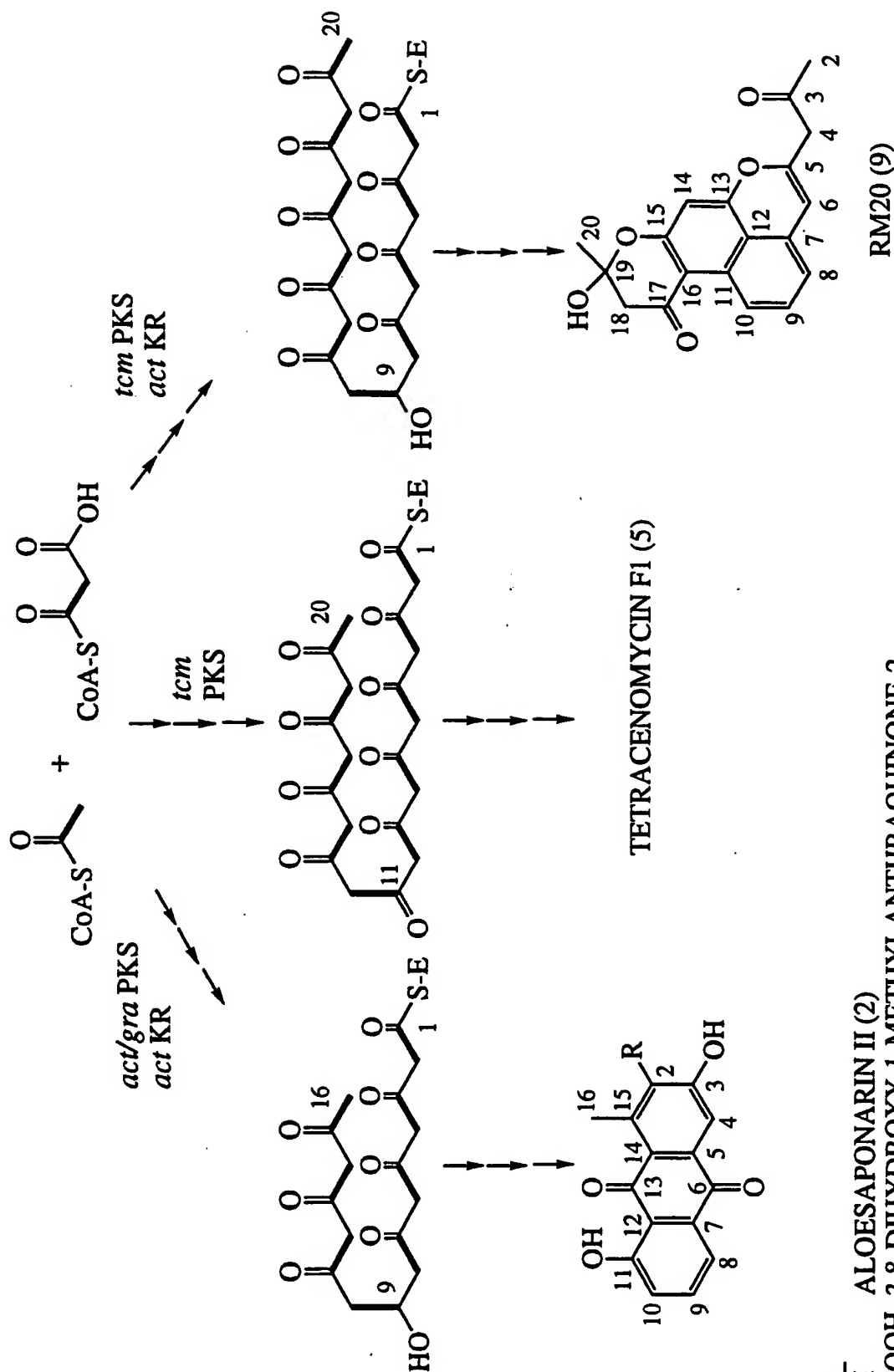
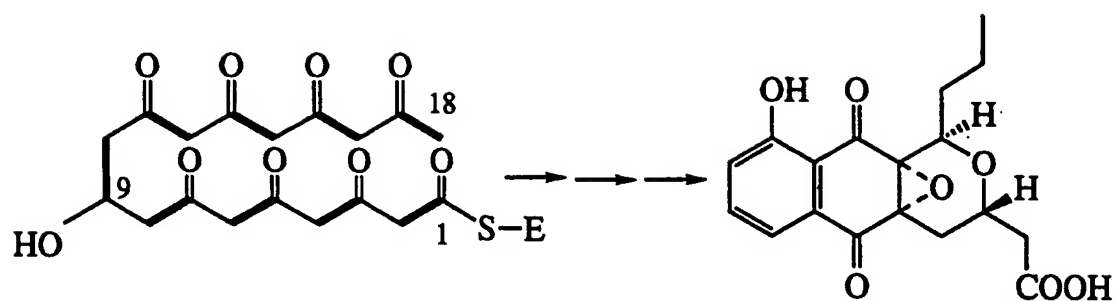
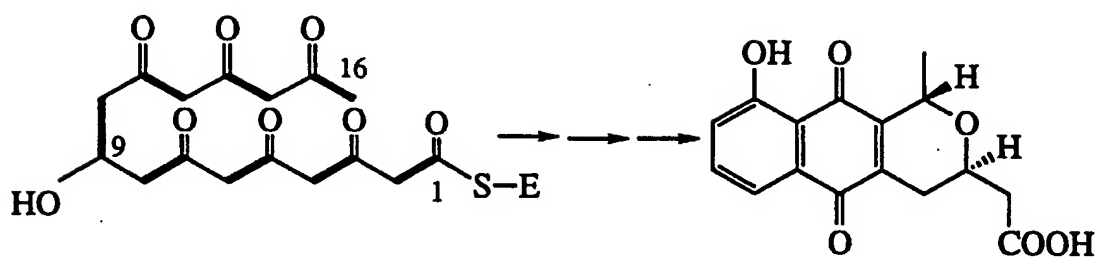


FIG. 6

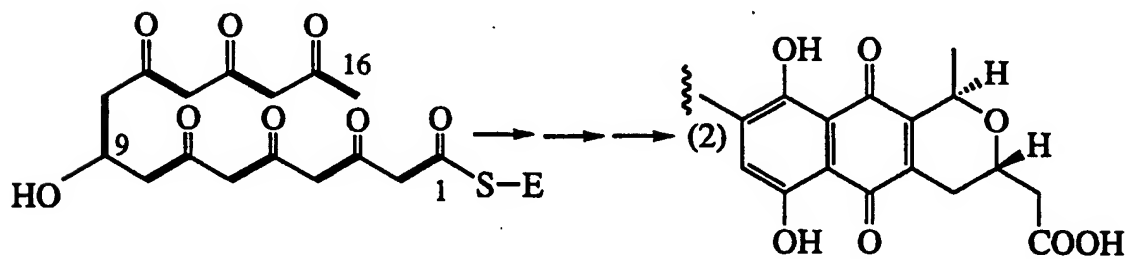
R-H ALOESAPONARIN II (2)  
 -COOH 3,8-DIHYDROXY-1-METHYLANTHRAQUINONE-2-CARBOXYLIC ACID (1)



FRENOLICIN B (7)

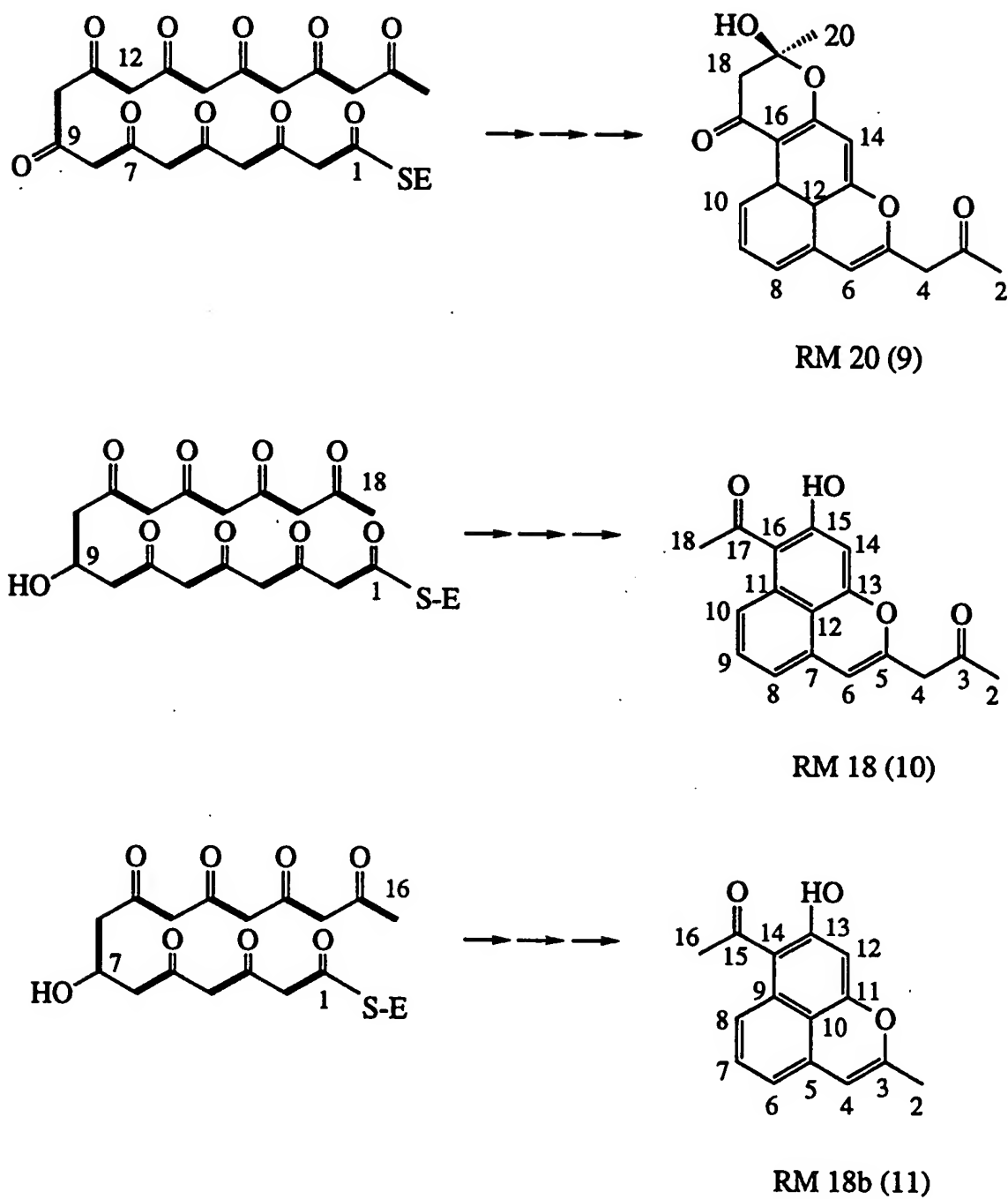


NANAOMYCIN A (8)



ACTINORHODIN (3)

FIG. 7

**FIG. 8A**



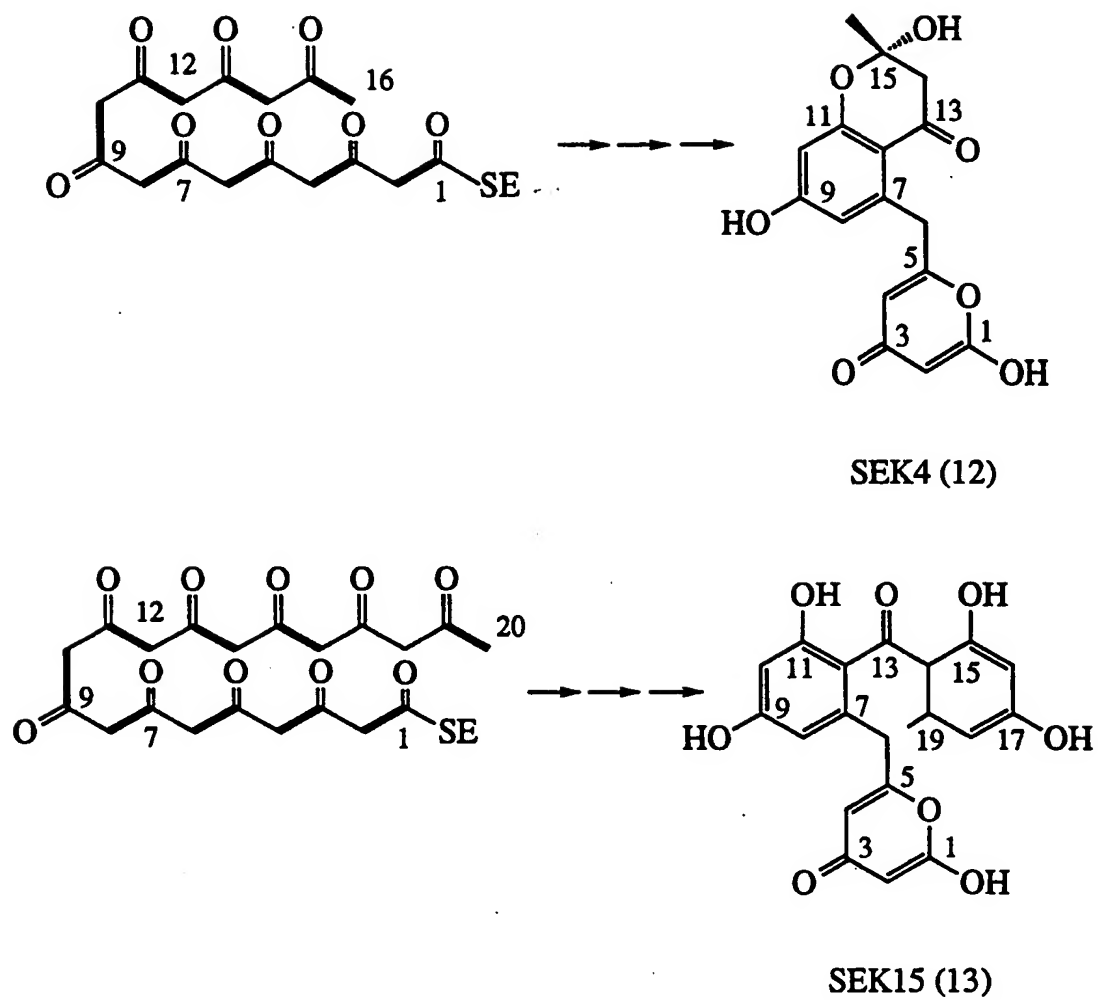
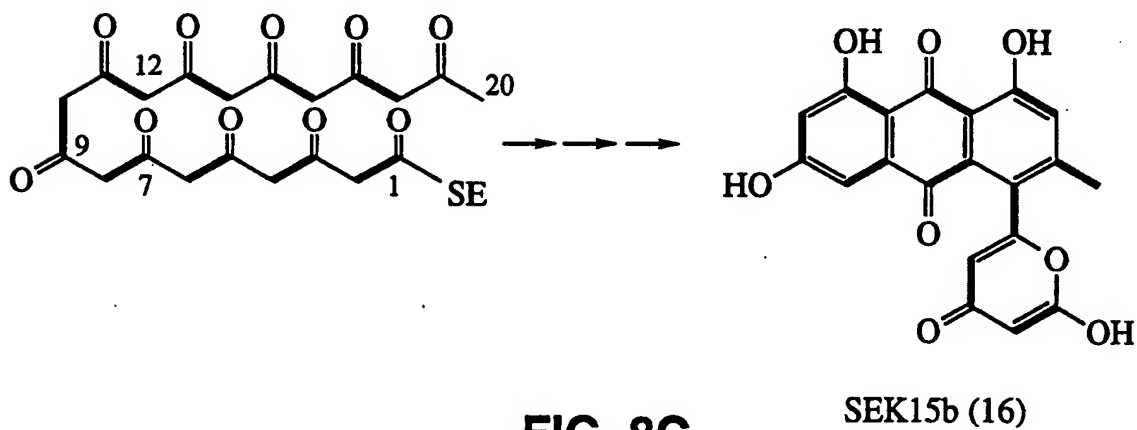
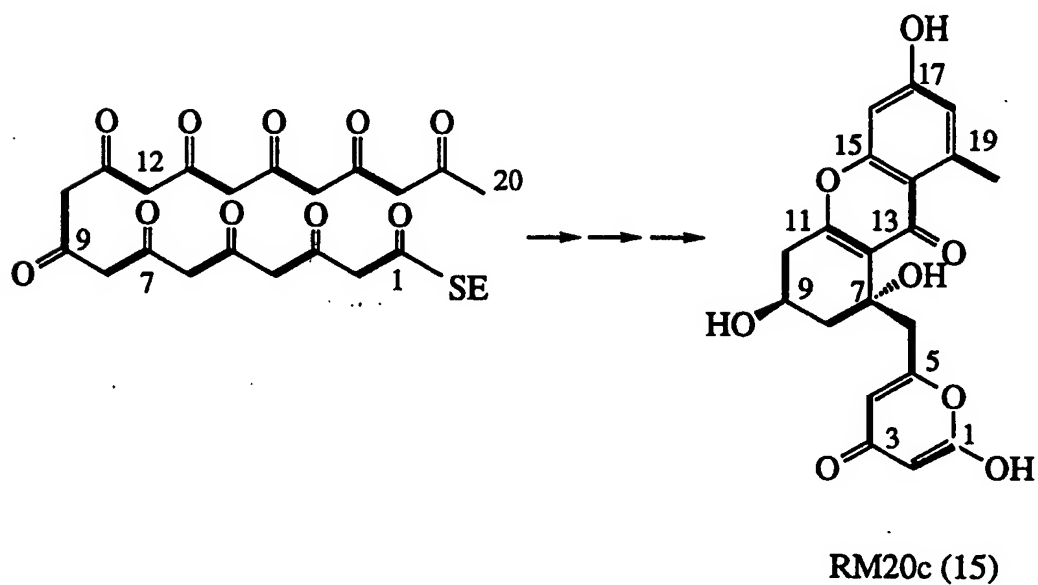
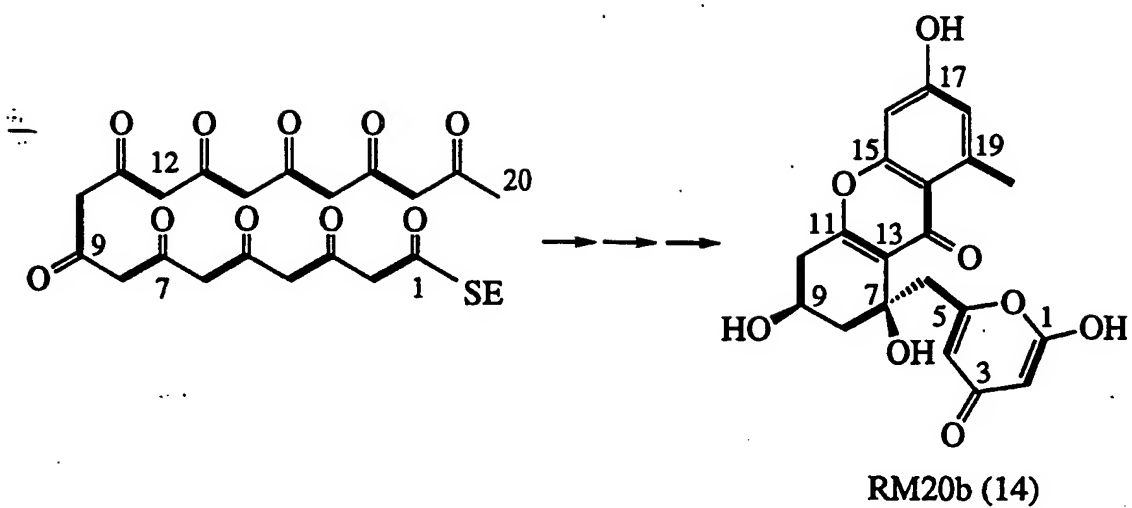


FIG. 8B



**FIG. 8C**

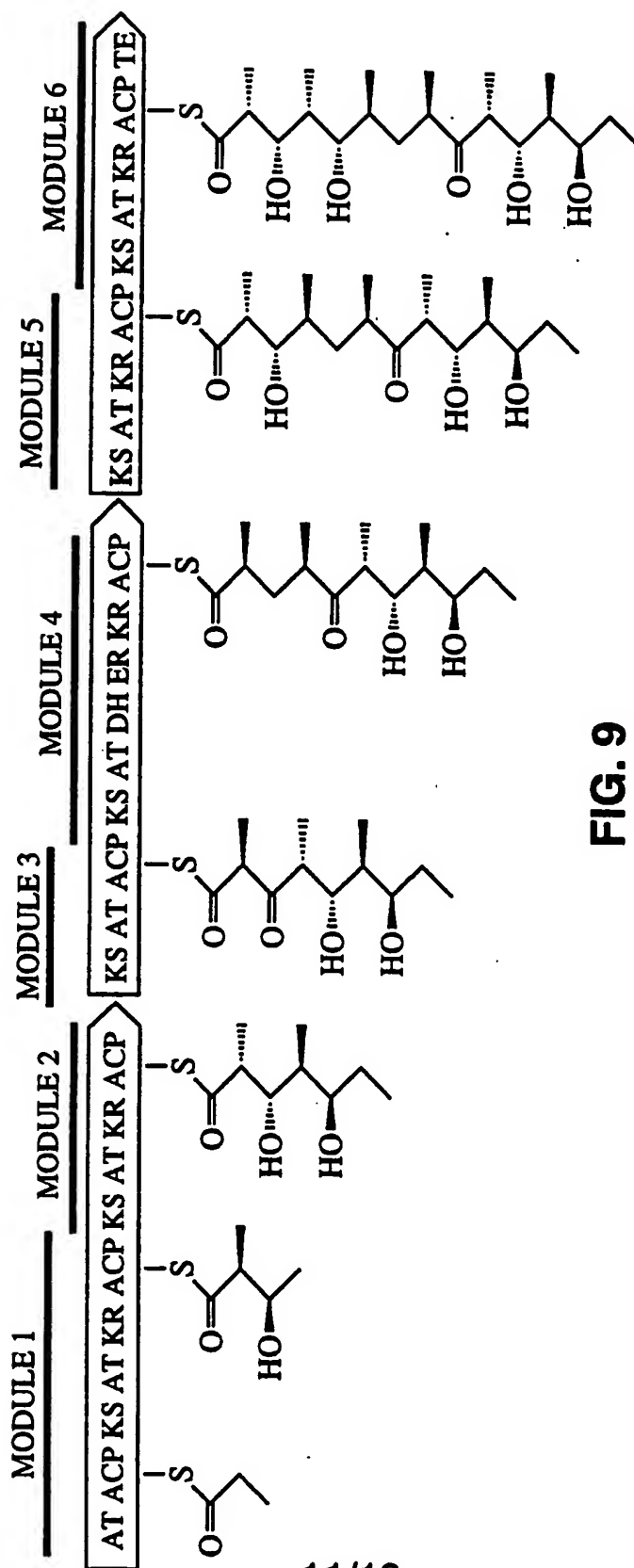


FIG. 9

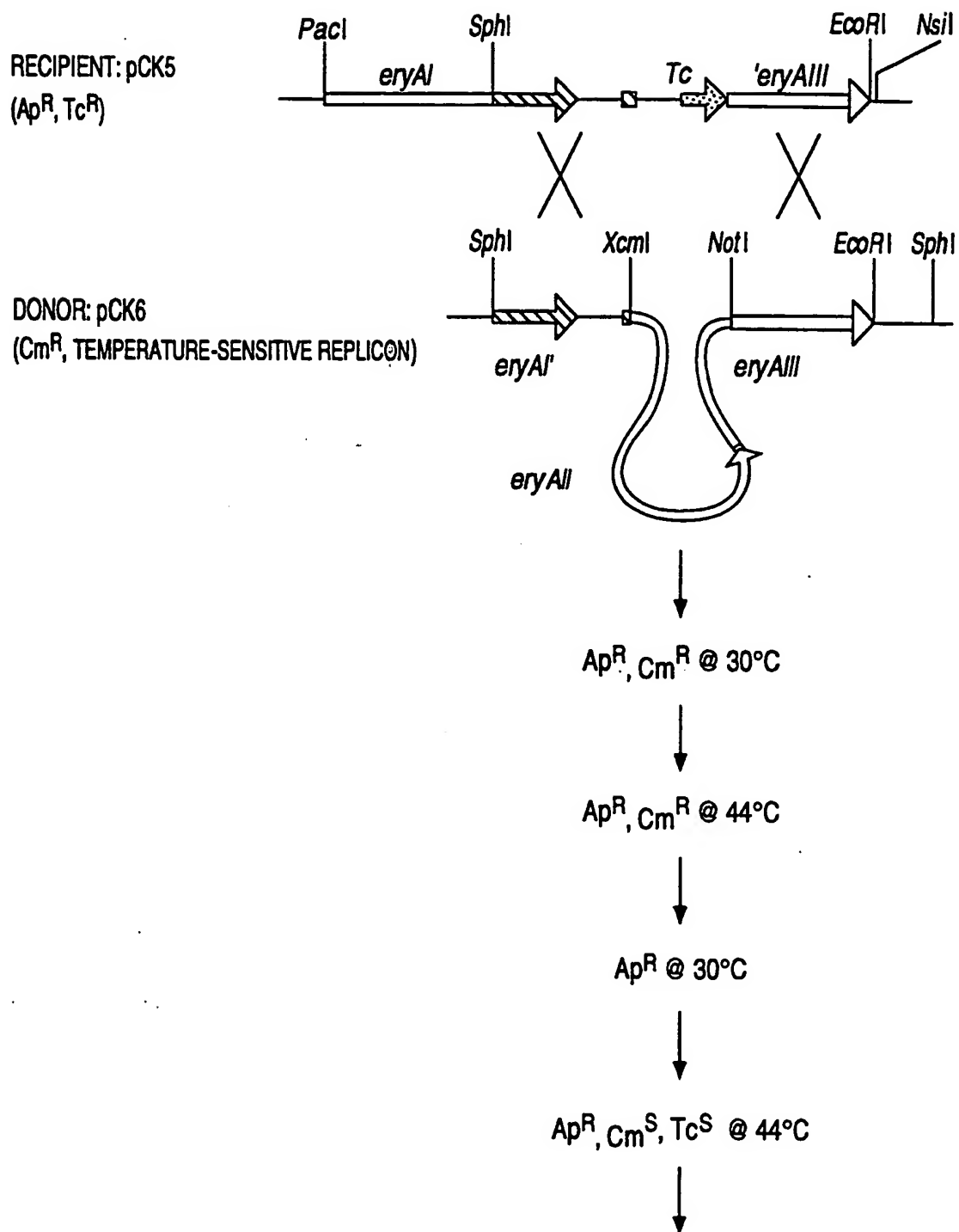


FIG. 10

12/13

SUBSTITUTE SHEET (RULE 26)

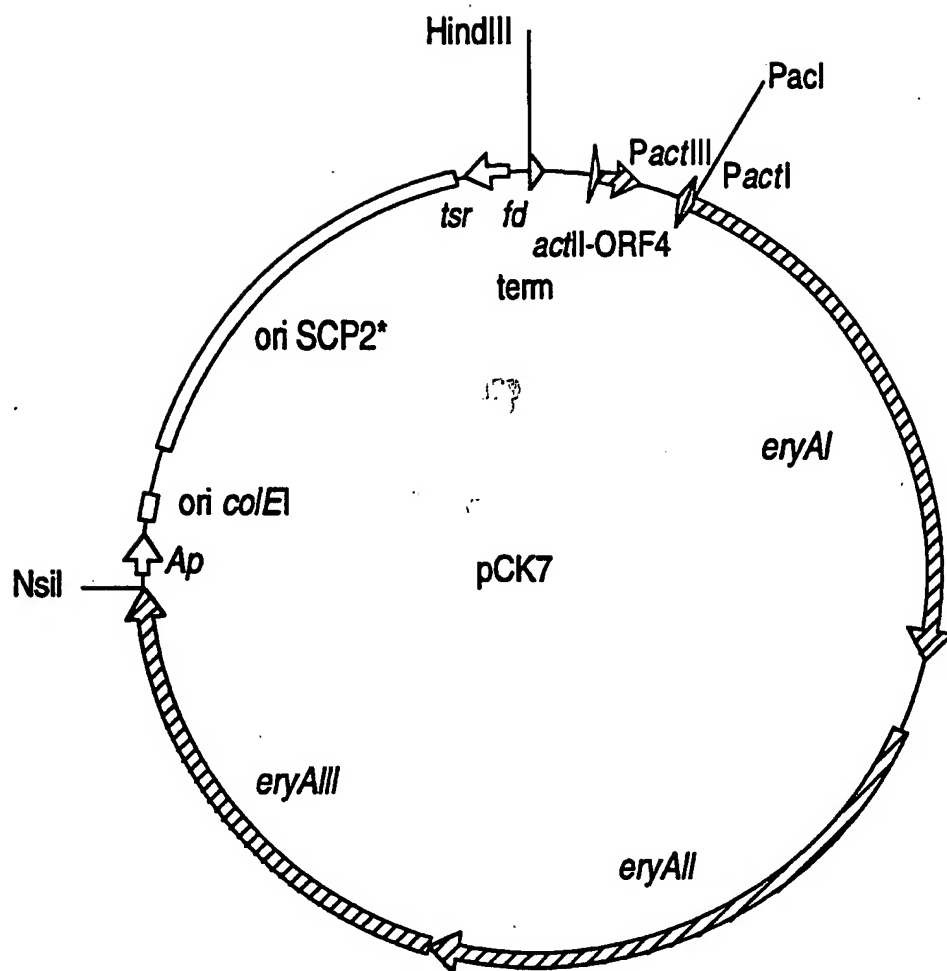


FIG. 11

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/10643

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/118, 125, 127, 252.35, 253.5, 320.1; 549/385, 387, 388, 392; 552/208

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ----- Y, P	Biochemistry, Vol. 33, No. 31, issued 1994, Fu et al., "Engineered biosynthesis of novel polyketides: Stereochemical course of two reactions catalyzed by a polyketide synthase", pages 9321-9326, see the entire document.	1-16, 18, 22, 26, 61, 62 ----- 17, 19-21, 23- 25, 27-30, 55, 56
X, P ----- Y, P	Science, Vol. 262, issued 03 December 1993, McDaniel et al., "Engineered biosynthesis of novel polyketides", pages 1546-1550, see the entire document.	1-18, 20, 22, 26, 27, 29, 45, 64 ----- 19, 21, 23-25, 28

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 05 DECEMBER 1994	Date of mailing of the international search report DEC 28 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ERIC GRIMES <i>E. Grimes</i> Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US94/10643

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Tetrahedron, Vol. 47, No. 31, issued 1991, Floss et al., "Genetic engineering of hybrid antibiotics - a progress report", pages 6045-6058, see pages 6046-6049.	1-73
Y	Nature, Vol. 314, issued 18 April 1985, Hopwood et al., "Production of 'hybrid' antibiotics by genetic engineering", pages 642-644, see the entire document.	1-73
Y	Journal of Bacteriology, Vol. 175, No. 8, issued April 1993, Khosla et al., "Genetic construction and functional analysis of hybrid polyketide synthases containing heterologous acyl carrier proteins", pages 2197-2204, see the entire document.	1-73
Y	Gene, Vol. 115, issued 1992, Donadio et al., "Biosynthesis of the erythromycin macrolactone and a rational approach for producing hybrid macrolides", pages 97-103, see the entire document.	1-73
Y	Journal of Bacteriology, Vol. 174, No. 19, issued October 1992, Sherman et al., "Functional replacement of genes for individual polyketide synthase components in <i>Streptomyces coelicolor</i> A3(2) by heterologous genes from a different polyketide pathway", pages 6184-6190, see the entire document.	1-73
Y	Nature, Vol. 309, issued 31 May 1984, Malpartida et al., "Molecular cloning of the whole biosynthetic pathway of a <i>Streptomyces</i> antibiotic and its expression in a heterologous host", pages 462-464, see the entire document.	1-73
Y	HERSHBERGER ET AL., GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL MICROORGANISMS, published 1989 by the American Society for Microbiology (Washington, DC), pages 68-84, see especially pages 78-82.	1-73
A	Annual Review of Microbiology, Vol. 47, issued 1993, Katz et al., "Polyketide synthesis: Prospects for hybrid antibiotics", pages 875-912.	1-73

**A. CLASSIFICATION OF SUBJECT MATTER:**

IPC (6):

C07D 311/00, 311/78, 493/00; C12N 1/21, 15/74; C12P 15/00, 17/06, 17/16

**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

435/118, 125, 127, 252.35, 253.5, 320.1; 549/385, 387, 388, 392; 552/208

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Dialog

search terms: polyketide, synthase, pks, streptomyces, ketosynthase, ketoreductase, cyclase, dehydratase, hybrid, heterologous, host, recombinant, rm20, sek4, sek15

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-25, 27-30 and 64-73, drawn to vectors and host cells comprising a polyketide synthase gene cluster and a method of using said host cells.

Group II, claims 26 and 31-63, drawn to polyketides.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The technical feature of Group I is genes encoding polyketide synthesis genes. The technical feature of Group II is polyketides. The claims thus do not share a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.